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THE UNIVERSITY OF ALBERTA

Characterization of the Cytochrome *c* Deficient Mutant *Cyc-1-12* of *Neurospora crassa*

by

Drell Allan Bottorff

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF Master of Science

Genetics

EDMONTON, ALBERTA
Spring, 1990

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Characterization of the Cytochrome *c*-Deficient Mutant *Cyc-1-12* of *Neurospora crassa* submitted by Drell Allan Bottorff in partial fulfilment of the requirements for the degree of Master of Science.

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This thesis is dedicated to LeRoy and Florence Bottorff for their love and support and to Michele for helping and encouraging me through difficult moments.

Abstract

The *cyt-12-1* mutant is characterized by a deficiency of cytochrome c and a partial deficiency of cytochrome aa_3, while the *cyc-1-1* mutant is deficient only in cytochrome *c*. The $cyc\text{-}1^{+}$ gene was isolated by sib-selection based on the rescue of the slow-growth phenotype of *cyc-1-1* using a cosmid library of *N.crassa* DNA. Others in our laboratory isolated the $cyc\text{-}12^{+}$ gene using identical techniques. Since the phenotypes of these mutants are slightly different, it was originally thought that *cyt-12-1* and *cyc-1-1* affected different genes. However, three observations were made which demonstrate that the mutants are, in fact, allelic. First, heterokaryon analysis revealed that *cyt-12-1* and *cyc-1-1* do not complement. Second, it was found that the cosmid containing the $cyt\text{-}12^{+}$ gene was capable of rescuing the *cyc-1-1* mutant and vice versa. Third, a comparison of the $cyt\text{-}12^{+}$ containing cosmid with the $cyc\text{-}1^{+}$ containing cosmid, demonstrated that both cosmids contain the same gene capable of rescuing both mutants.

DNA sequence analysis showed that the $cyt\text{-}12^{+}$ gene encodes the cytochrome *c* structural gene and that the gene contains two introns, one located near the amino terminus and the other near the carboxyl terminus. DNA sequence analysis of the *cyt-12-1* mutant allele revealed two mutations, a deletion of a G residue at -363 and a transition at +579. This G/C to A/T transition affects amino acid #10 of the *N.crassa* cytochrome *c* protein by causing the replacement of

the wild-type glycine residue with an aspartic acid. The glycine in the wild-type protein is thought to be important for the formation of an alpha-helix near the amino-terminus of the protein (Hampsey *et al.*, 1986).

Northern analysis of cytochrome *c* transcripts revealed that *cyt-12-1* cytochrome *c* mRNA comigrates with that of the wild-type strain and that the levels of cytochrome *c* transcripts from the two strains are not grossly different. Immunoprecipitation studies demonstrated that *cyt-12-1* apocytochrome *c* mRNA can be translated in rabbit reticulocyte lysates and that *cyt-12-1* mitochondria are almost completely devoid of immunologically detectable holocytochrome *c*.

In light of the above findings, a model is proposed to explain the lack of holocytochrome *c* in *cyt-12-1* and the possible relationship of the cytochrome *c* deficiency to that of cytochrome *aa*$_3$. The finding that the *cyt-12-1* mutation is allelic to cyc-1-1 and that the cyt-12-1 gene encodes the structural gene for cytochrome *c*, suggests that *cyt-12-1* should be renamed *cyc-1-12*.

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Abbreviations

Ap^{R}	ampicillin resistance
Bn^{R}	benomyl resistance
bisacrylamide	N,N'-methylenebisacrylamide
bp	base pair
bromphenol blue	3',3'',5',5''-tetrabromophenol-sulfonphthalein
BSA	bovine serum albumin
Ci	Curie
cpm	counts per minute
Da	Daltons
dATP	2'-deoxyadenosine 5'-triphosphate
ddATP	2',3'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddCTP	2',3'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
ddGTP	2',3'-deoxyguanosine 5'-triphosphate
dITP	2'- deoxyinosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddTTP	2',3'-deoxythymidine 5'-triphosphate
dH20	distilled water
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*

EDTA	ethylenediaminetetraacetic acid, disodium salt
IPTG	isopropyl-beta-D-thiogalactoside
kb	kilobase pairs
kDa	kiloDaltons
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced form)
N.crassa	*Neurospora crassa*
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
Tc^R	tetracycline resistance
Tris	Tris(hydroxmethyl) aminomethane
triton X-100	octyl phenoxy polyethoxyethanol
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

I. Introduction

Mitochondrial Genetics

The study of the components and regulatory mechanisms involved in the mitochondrial cytochrome system began with the discovery of maternally inherited respiratory-deficient mutants in yeast (Ephrussi and Hottinguer, 1951) and in *Neurospora* (Mitchell and Mitchell, 1952; Mitchell *et al*., 1953). The finding that mitochondria contained DNA (Luck and Reich, 1964) and that the pattern of inheritance of these cytoplasmic organelles was associated with many abnormal phenotypes in *Neurospora* (Reich and Luck, 1966) suggested that some metabolic functions were encoded by mitochondria. The transmission of the *Neurospora crassa* cytoplasmic [*abn-1*] mutant phenotype to normal strains by microinjection of cytoplasm from [*abn-1*] cultures, provided evidence supporting the genetic importance of mitochondria (Garnjobst *et al*., 1965; Diacumakos *et al*., 1965).

It is now known that virtually all mitochondrial genomes specify the same number of proteins, even though extensive size variation exists in mitochondrial DNAs among various species (Sederoff, 1984; Mulligan and Walbot, 1986). Mitochondrial DNA only encodes a few electron transport chain proteins: apocytochrome *b*, three components of the cytochrome aa_3 complex, and some subunits of the mitochondrial ATPase and NADH dehydrogenase complex (Sederoff, 1984; Breitenberger and RajBhandary, 1985; Hartl *et al.*, 1989). The remaining proteins are encoded by nuclear genes and have to be transported into the appropriate submitochondrial compartment.

Mutants have been isolated, most notably in yeast and *N.crassa*, affecting various aspects of respiratory function. The fewer number of respiratory mutants isolated in *Neurospora* reflects the fact that yeast is a facultative aerobe, while *Neurospora* is an obligate aerobe. Thus, an effective selection scheme for respiratory mutants does not exist for *Neurospora*. Lesions that completely eliminate respiration in yeast are not lethal, because yeast has the ability to grow by fermentation. Yeast respiratory mutants can be easily detected on a medium containing high levels of glycerol and low concentrations of glucose, since respiratory deficient mutants stop growing when the stock of glucose is depleted, while wild-type cells continue to grow by utilizing the glycerol as a carbon source (Tzagoloff, 1982). However, *Neurospora* lacks the ability to grow anaerobically, so that mutations completely destroying respiratory functions would be expected to be fatal. Thus, respiratory mutants observed in *Neurospora* are due to a reduction, rather than a total loss, of respiratory function.

Several techniques have been utilized solely or in various combinations, in an attempt to select for respiratory mutants of *Neurospora*. The filtration enrichment method (Pittenger and West, 1979; Bertand *et al.*,

1977) used in combination with the inositol-less death selection method (Pittenger and West, 1979) or with the tetrazolium dye reduction test (Bertrand *et al.*, 1977) have yielded some respiratory mutants.

Cytochrome *c*

Cytochrome *c*, a component of the respiratory chain (Figure 1), acts as an electron carrier between cytochrome *c* reductase and cytochrome *c* oxidase on the outer face of the inner mitochondrial membrane (Chanse and Hess, 1959; Estabrook, 1961; Hackenbrock and Hammon, 1975; DePierre and Ernster, 1977). The ease of isolating cytochrome *c* protein allowed the characterization of cytochrome *c* and many mutants affecting the protein before the era of recombinant DNA (Sherman *et al.*, 1985; Hampsey *et al.*, 1986). Protein sequence analysis of cytochrome *c* from *N.crassa* revealed that cytochrome *c* is a small protein, only 107 amino acids in length (Heller and Smith, 1966; Lederer and Simon, 1974). Cytochrome *c* is one of the many mitochondrial products that undergo various forms of post-translational modification. Cytochrome *c* of *N.crassa* does not contain a cleavable amino-terminal prepiece (Zimmermann *et al.*, 1979; Hennig and Neupert, 1983; Harmey and Neupert, 1985), in contrast to both iso-cytochromes *c* of yeast, known to have their amino-terminal methionines cleaved by a general methionine aminopeptidase (Sherman *et al.*, 1985). Apocytochrome *c*, the form of the protein without the heme group attached, is encoded by a nuclear gene and is synthesized on free polysomes before being released as a soluble protein into the cytosol (Sherman *et al.*, 1966; Verdiere and Petrochilo, 1975; Korb and Neupert, 1978; Sherman *et al.*, 1978; Montgomery *et al.*, 1978; Smith *et al.*, 1979; Montgomery *et al.*, 1980). It has been determined that apocytochrome *c* in yeast and *Neurospora* contains an unusual N-trimethyl-L-lysine residue, instead of the normal lysine at position 72 (DeLange *et al.*, 1969). *In vitro* import experiments in yeast have shown that methylated apocytochrome c has a higher affinity for mitochondria than the unmethylated form (Polastro *et al.*, 1978; Hartl *et al.*, 1989). Apocytochrome *c* is recognized by a unique mitochondrial transport system and imported into the intermembrane space (Korb and Neupert, 1978; Zimmermann *et al.*, 1981). Several studies have revealed that cytochrome *c* import is concommitant with cytochrome *c* heme attachment in *N.crassa* and *S.cerevisiae* (Hennig and Neupert, 1981; 1983; Hennig *et al.*, 1983; Nicholson *et al.*, 1988; Stuart, 1989). The heme prosthetic group is attached to partially inserted apocytochrome *c* by the formation of thioether linkages formed between the heme vinyl groups and the thiols of cysteines 14 and 17 of *Neurospora* apocytochrome *c* and cysteines 18 and 21 of yeast iso-1 and iso-2 apocytochrome *c* (Korb and Neupert, 1978; Neher *et al.*, 1980; Basile *et al.*, 1980; Veloso *et al.*, 1981; Dumont *et al.*, 1987).

Cytochrome *c* Mutants in Yeast

The expression of cytochrome *c* in yeast has been shown to be regulated by the level of mitochondrial metabolism (Slonimski *et al.*, 1965; Zitomer and Hall, 1976; Zitomer and Nichols, 1978; Boss *et al.*, 1980; Siemens *et al.*, 1980), which suggests that lesions affecting a variety of mitochondrial functions may directly or indirectly influence cytochrome *c* levels in the respiratory chain.

S.cerevisae, contains two distinct forms of cytochrome *c*. The major form, iso-1-cytochrome *c*, is encoded by the *CYC1* gene, and the minor form, iso-2-cytochrome *c*, is encoded by the *CYC7* gene (Smith *et al.*, 1979; Boss *et al.*, 1981; Sherman *et al.*, 1981). The fact that various mutations localized in the upstream regions of *CYC1* or *CYC7* cause either the overproduction or deficiency of their gene products, suggests that a complex regulatory system exists to control the expression of iso-cytochromes *c* in yeast (Sherman, 1964; Sherman *et al.*, 1965; Lawrence *et al.*, 1975; Downie *et al.*, 1977a and 1977b; Sherman *et al.*, 1978; Sherman and Stewart, 1978; Sherman *et al.*, 1981; Matner and Sherman, 1982).

Mutations at two other loci, *CYC2* and *CYC3*, that are unlinked to *CYC1* and *CYC7* , reduce the levels of both iso-1-cytochrome *c* and iso-2-cytochrome *c*, although these mutants have higher than normal levels of apo-iso-1-cytochrome *c* and apo-iso-1-cytochrome *c* (Sherman, 1964; Sherman *et al.*, 1965; Rothstein and Sherman, 1980; Mather and Sherman, 1982).

Wild-type yeast cultures contain small amounts of apo-iso-2-cytochrome *c* and no detectable apo-iso-1-cytochrome *c*. The observations that both apo-cytochromes *c* are detectable in *cyc2* and *cyc3*, that *cyc3* is completely deficient of both iso-cytochromes *c*, and that *cyc2* partially lacks iso-1 and iso-2-cytochrome *c*, suggested that these mutations affect post-translational processing steps of cytochromes *c* (Matner and Sherman, 1982). The severity of the cytochromes *c* deficiency observed in *cyc3* mutants suggested that this mutant probably directly affects cytochrome *c* biogenesis. It was later determined that the *CYC3* gene encodes the enzyme cytochrome *c* heme lyase (CCHL), which attaches the heme group to apocytochrome *c* (Harmey and Neupert, 1985; Dumont *et al.*, 1987). Strangely, some *cyc3* mutants also lack cytochrome aa_3. It has been proposed that this deficiency of cytochrome aa_3 is a secondary effect due to the absence of cytochrome *c* (Sherman *et al.*, 1965; Reilly and Sherman, 1965), since *cyc1/cyc7* double mutants are also deficient in cytochrome aa_3 (Downie *et al.*, 1977a).

Even the most severe *cyc2* mutants still contain 10% of the normal levels of both iso-cytochromes *c*. Thus, it has been speculated that the *CYC2* protein is not directly involved in cytochrome *c* metabolism, but could be required for efficient import of apocytochromes *c*, or the enhancement of heme attachment activity (Matner and Sherman, 1982). However, the exact role of *cyc2* has not been determined.

Other yeast mutants, affecting one or both iso-cytochromes *c*, have been studied to a limited degree. *Hem1* mutants are in some senses phenotypically similar to cyc3 mutants in that they lack both iso-cytochromes *c*, but contain high levels of apo-iso-2-cytochrome *c*. However, these *hem1* mutants also lack all other cytochromes and hemeproteins and were found to be deficient in delta-aminolevulinic acid synthetase, an important enzyme of the porphyrin biosynthetic pathway (Matner and Sherman, 1982; Keng and Guarente, 1987). Three other cytochrome *c* mutants, *cyc4*, *cyc5*, and *cyc6* ,are slightly deficient of iso-1-cytochrome *c* and iso-2-cytochrome *c* (Sherman, 1964; Sherman *et al.*, 1965; Woods *et al.*, 1975). Woods *et al.* (1975) determined that *cyc4* mutants are also defective in porphyrin metabolism. The effects of *cyc5* and *cyc6* on the levels of cytochrome *c* are very weak. In fact, the amount of variation in cytochrome *c* content in various polymorphic strains of yeast is as great as that seen in the mutant strains *cyc5* and *cyc6*. Therefore, it is thought that both mutants indirectly affect cytochrome *c* metabolism (Gilmore *et al.*, 1971; Rothstein and Sherman, 1980). Two mutants, *cyc8* and *cyc9*, cause the overproduction of iso-2-cytochrome *c* in addition to other pleiotrophic affects (Rothstein and Sherman, 1980; Sherman *et al.*, 1981). Complementation studies revealed that the *CYC9* gene is allelic to the *TUB1* gene, known to encode tubulin (Rothstein and Sherman, 1980). The tub1 mutant is characterized by a low frequency of mating, abnormally shaped cells, flocculent morphology, and the ability to incorporate exogeneous dTMP into DNA (Wickner, 1974; Rothstein and Sherman, 1980). These diverse mutant characteristics of cyc9 and tub1 reinforce the complex nature of these two regulatory mutants.

Regulation of Cytochrome *c* in Yeast

Upstream activation sequences (UAS) mediate specific control of several yeast genes. The *CYC1* gene contains two distinct UAS sites, UAS1 and UAS2, that can function independently to activate transcription (Pinkham and Guarente, 1985; Pfeifer *et al.*, 1987), while the *CYC7* locus only contains one UAS (Pfeifer *et al.*, 1987). Several studies have shown that *CYC1*, *CYC7*, and the *CTT1* gene, which encodes the soluble catalase T protein that presumably neutralizes superoxide radicals, are regulated by various physiological signals including oxygen, heme, and catabolite repression (Clavilier *et al.*, 1976; Guarente and Mason, 1983; Guarente *et al.*, 1984; Winkler *et al.*, 1988). Mutation and protection studies have shown that $CYC1_{UAS1}$, $CYC1_{UAS2}$, $CYC7_{UAS}$, and $CTT1_{UAS}$ consist of at least two functional components (regions A and B) which interact synergistically to provide full expression (Forsburg and Guarente, 1988).

Heme-deficient cells (either cells mutant in heme biosynthesis grown on non-heme supplemented media or wild-type cells grown anaerobically) can not respire and also lack all cytochromes (Ephrussi and Slonimski, 1950; Gollub *et al.*, 1977; Guarente and Mason, 1983). It was determined that under anaerobic growth conditions or the absence of heme, both upstream activation sites of *CYC1* are inactive (Pinkham andd Guarente, 1985). However, under heme-sufficient conditions in glucose media, activation of transcription of *CYC1* by UAS1 is 10 fold greater than that of UAS2, whereas in lactate media UAS2 is derepressed an additional 100 fold, while UAS1 is derepressed an additional 10 fold (Pinkham and Guarente, 1985). Thus, when cells are grown in glucose the basal levels of transcription of CYC1 are essentially activated by UAS1, while induction in non-fermentable carbon sources is mediated equally by both UAS1 and UAS2 (Forsburg and Guarente, 1988).

In vitro studies have shown that a transciptional activator, the *HAP1* gene product (formerly called *CYP1*), binds to region B of *CYC1*$_{\text{UAS1}}$, *CYC7*$_{\text{UAS}}$, and CTT1$_{\text{UAS}}$, and that binding is greatly stimulated by heme (Guarente and Mason, 1983; Lalonde *et al.*, 1986; Pfeifer *et al.*, 1987; Cerdan and Zitomer, 1988; Winkler *et al.*, 1988). Interestingly, the pattern of *HAP1* major and minor groove contacts were found to be similar among the B regions of *CYC1*$_{\text{UAS1}}$, *CYC*$_{\text{UAS}}$, and *CTT1*$_{\text{UAS}}$ sites (Winkler *et al.*, 1988).

It was also determined that another yeast factor, designated *RC2*, binds to a site overlapping region B of *CYC1*$_{\text{UAS1}}$ (Arcangioli and Lascure, 1985). However, the exact role of *RC2* in the regulation of iso-1-cytochrome *c* expression still remains unknown.

Another factor, known as *RAF*, was found to bind to region A of $CYC1_{UAS1}$ (Lalonde *et al.*, 1986). It is thought that bound *RAF* is a requirement for *HAP1* recognition and that the complex of *RAF* and *HAP1* is necessary for the transcriptional activation of *CYC1* by UAS1 (Pfeifer *et al.*, 1987).

Although, oxygen regulation of CYC1, CYC7, and CTT1 is not fully understood, it has been shown that an enzyme of the heme biosynthetic pathway in yeast, delta-aminolevulinic synthetase, requires molecular oxygen for induction (Mattoon et al., 1979). It has been suggested that oxygen may also directly affect the expression of these genes by increasing the affinity of HAP1 to upstream activation sequences, similiar to the affect of intracellular levels of heme (Cerdan and Zitomer, 1988).

The molecular basis of catabolite repression is not completely understood in yeast (Guarente *et al.*, 1984). Guarente *et al.* (1984) proposed that catabolite repression of UAS1 is dictated by intracellular heme levels, since derepression of UAS1 in lactate is triggered by an increase in levels of intracellular heme. This model is in agreement with the observation that the rate limiting enzyme, delta-aminolevulinate dehydratase, of the porphyrin biosynthetic pathway, is also regulated by catabolite repression (Mahler and Lin, 1978; Guarente *et al.*, 1984). Other evidence suggests that UAS2 is highly regulated by catabolite repression and is derepressed approximately 50 fold when cells are shifted from a glucose medium to a non-fermentable carbon source (Hahn *et al.*, 1988).

In cells grown on non-fermentable carbon sources two regulatory products, *HAP2* and *HAP3*, are required for the activation of the *CYC1* upstream activation site UAS2, *HEM1*, and some other yeast genes encoding respiratory functions, including other cytochromes and heme biosythesis enzymes (Pinkham *et al.*, 1987). In fact, the level of HAP2 mRNA is 5 fold higher in cells grown in medium containing a non-fermentable carbon source than in glucose medium (Pinkham and Guarente, 1985). However, the notion that HAP2 is the direct regulator of UAS2 is complicated by the fact that mutations in either *HAP2* or *HAP3* prevent growth on non-fermentable carbon sources and completely abolish *CYC1*$_{\text{UAS2}}$ activity (Hahn *et al.*, 1988) Strangely, it was observed that the *HAP3* locus encodes divergent overlapping transcripts (Hahn *et al.*, 1988). The antisense transcript has been implicated in the regulation of *HAP3* synthesis and/or required for the activity of an activation complex containing *HAP2* and *HAP3* (Hahn *et al.*, 1988). Although it has been observed that *HAP3* binds to *CYC1*$_{\text{UAS2}}$ after *HAP2* and requires *HAP2*, it has not yet been determined which protein makes the critical contacts to the upstream activation sequence (Olesen *et al.*, 1987).

Neurospora Cytochrome Mutants

The regulation of cytochrome *c* production in *Neurospora* is not as well understood as in yeast. However, certain mutants of the organism, deficient in cytochrome *c* and other cytochromes, have been implicated in a number of rather complex control pathways. For this reason a general discussion of relevant cytochrome deficient mutants is given prior to a specific discussion of cytochrome *c* mutants. The nomenclature of the *N.crassa* mutants discussed is as proposed by Bertrand *et al.* (1977). Under this scheme nuclear mutants designated "cyb" are deficient in cytochrome *b*, "cya" refers to strains deficient in cytochrome aa_3, "cyc" mutants lack cytochrome *c*, and "cyt" mutants are deficient in more than one cytochrome. *N.crassa* cytoplasmic mutants are designated by square brackets.

The cytochrome aa_3 deficiencies observed in *cya-3*, [*mi-3*] (deficient of cytochrome aa_3), and *cyt-2-1* (lacks cytochromes aa_3 and *c*) can be suppressed by low concentrations of antimycin A (Figure 1), an inhibitor that affects electron transport in the cytochrome bc_1 region of the respiratory chain (Mitchell *et al.*, 1953; Bertrand and Collins, 1978). Even wild-type cultures grown in the presence of low concentrations of antimycin A seem to have increased levels of cytochrome aa_3 (Bertrand and Collins, 1978; Bottorff and Nargang, unpublished results). The [*mi-3*] extranuclear mutant and *cyt-2-1*, are characterized by the accumulation of a larger cytochrome oxidase subunit 1 polypeptide (Bertrand and Collins, 1978; Bertrand and Werner, 1979), while [*mi-3*] is also deficient in immunoprecipitable cytochrome oxidase subunit 2 (Bertrand and Werner, 1977). DNA sequence analysis of the [*mi-3*] *oxi-3* gene revealed that a missense mutation of the mature subunit 1 polypeptide of cytochrome oxidase was most likely responsible for the [*mi-3*] phenotype and suggested that the subunit 2 deficiency of [*mi-3*] is likely a secondary affect (Lemire and Nargang ,1986). The mutant alleles of three nuclear genes *cyb-1-1*, *cyb-2*, and suppressor of [*mi-3*], designated *su*[*mi-3*], have been shown to partially or fully suppress the cytochrome aa_3 deficiency of [*mi-3*] (Bertrand *et al.*, 1976; Bertrand and Collins, 1978). However, only *cyb-1-1* can suppress the cytochrome aa_3 deficiency of *cyt-2-1* (Bertrand and Collins, 1978). Surprisingly, *cyt-2-1/cyb-1-1* double mutants contain cytochrome *b*, but [*mi-3*]*cyb-1-1* double mutants lack cytochrome *b* (Bertrand *et al.*, 1977; Bertrand and Collins, 1978). It has been suggested that the effect of *cyb-1-1* and *cyb-2-1* on [mi-3] and of *cyb-1-1* on *cyt-2-1* may be similar to the action of antimycin A on the cytochrome bc_1 segment of the electron transport chain (Bertrand and Collins, 1978; Bertrand, 1980).

A model involving two regulatory circuits was formulated to account for the results discussed above (Bertrand and Collins, 1978). One regulatory circuit involving the loci, *cyt-2-1*, [*mi-3*], and *cya-3*, was suggested as responsible for the constitutive production of cytochrome oxidase, since lesions in these genes lead to a deficiency in cytochrome *aa*$_3$. The second circuit was thought to control the induction of cytochrome oxidase in cultures of *cya-3*, *cyt-2-1* and [*mi-3*] supplemented with antimycin A, because a blockage of electron transport in the cytochrome *bc*$_1$ region of the respiratory chain appears to increase the levels of cytochrome *aa*$_3$ in these mutants. However, this model can not be entirely correct, since it has been recently shown that the primary defect in *cyt-2-1* directly affects cytochrome *c* metabolism (Nargang *et al*., 1988; Drygas *et al*., 1989). This implies that *cyt-2-1* does not directly affect a gene involved in cytochrome oxidase synthesis and its affect on cytochrome *aa*$_3$ levels is secondary.

Mutants of *N.crassa* Deficient in Cytochrome *c*

Only three mutants have been isolated and studied that have gross deficiencies of cytochrome *c* in *Neurospora crassa*. *Cyt-2-1* is characterized by its slow growth and deficiencies of cytochrome *c* and *aa*$_3$ (Mitchell *et al*., 1953) and was mapped to chromosome VI by linkage analysis and RFLP mapping (Bertrand *et al*., 1977; Drygas *et al*., 1989). Early studies focussed on the cytochrome *aa*$_3$ deficiency of the mutant and demonstrated that the cytochrome *aa*$_3$ deficiency of *cyt-2-1* could be alleviated under various conditions (discussed above). However, these conditions did not relieve the deficiency of cytochrome *c* (Bertrand and Collins, 1978).

Subsequently, it has been shown that the primary defect in *cyt-2-1* is a deficiency of cytochrome *c* heme lyase (Nargang *et al.*, 1988; Drygas *et al.*, 1989).

A cosmid clone containing, the *cyt-2*$^+$ gene, was isolated by sib-selection from a wild-type (74*A*) genomic *N.crassa* library, by rescuing the slow growth character of *cyt-2-1* (Drygas *et al.*, 1989). DNA sequence analysis of the *cyt-2*$^+$ gene revealed an open-reading frame of 346 amino acids interrupted by two introns that has homology to yeast cytochrome *c* heme lyase, encoded by the *CYC3* gene (Dumont *et al.*, 1987; Drygas *et al.*, 1989). The *cyt-2-1* mutant allele was cloned from a partial *cyt-2-1* genomic library, using the fragment containing the *cyt-2*$^+$ gene as the probe (Drygas *et al.*, 1989) Analysis of the sequence of the mutant gene revealed a two base deletion that changes the reading frame prior to generating an early stop codon (Drygas *et al.*, 1989). The finding that the *cyt-2* gene directly affects the biogenesis of cytochrome *c*, suggested that a severe deficiency of cytochrome *c* causes a reduction in cytochrome aa_3 production in *Neurospora*, which is similiar to observations in yeast (discussed above).

Another cytochrome c deficient *Neurospora* mutant, *cyc-1-1* (formally named RK3-21), was isolated as a temperature sensitive growth-rate mutant (Pittenger and West, 1979). Spectral analysis revealed that *cyc-1-1* was the first mutant deficient in only cytochrome *c* in *N.crassa* (Pittenger and West, 1979). *Cyc-1-1* contains normal amounts of cytochromes aa_3 and *b* at 25°C (Figure 2) (Pittenger and West, 1979), but partially lacks cytochrome aa_3 when grown at 37°C (D.A. Bottorff and F.E. Nargang, unpublished results).

The *N.crassa* mutant, *cyt-12-1*, was found to be completely deficient of cytochrome *c* and contained very low levels of cytochrome aa_3 when grown at 25°C (Figure 2) (Bertrand *et al.*, 1977). *Cyt-12-1*, when grown at 37°C, resembles *cyt-2-1* , since it lacks both cytochromes aa_3 and *c* (Mitchell *et al.*, 1953; D.A. Bottorff and F.E. Nargang, unpublished results). In early studies, the similiarity of the cytochrome defects observed in *cyt-2-1* and *cyt-12-1* suggested that both may affect similiar processes, despite the fact that they map to different loci. The elucidation of the defect in *cyt-12-1* forms the main part of the present work.

Mitochondrial Import of Proteins

Recently, considerable effort has gone into the analysis of the targeting and import mechanisms for mitochondrial proteins largely from studies in *S.cerevisiae* and *Neurospora crassa*. It has been estimated that the nucleocytoplasmic system contributes greater that 90% of the protein mass to mitochondria (Douglas *et al.*, 1986) with the remainder being mitochondrial gene products translated on mitochondrial ribosomes (Schatz and Mason, 1974; Schatz and Butow, 1983). Cytoplasmically synthesized proteins are ultimately translocated into one of the four mitochondrial compartments: outer membrane, intermembrane space, inner membrane, or matrix space (Doonan *et al.*, 1984). It is critical that proteins destined for the mitochondria are efficiently and accurately targeted to the proper mitochondrial compartment. The process of incorporating cytoplasmically translated proteins into mitochondria can be divided into a number of steps (discussed below), although some precursor proteins have the ability to by-pass certain steps (Hartl *et al.*, 1989).

Proteins destined for the mitochondria are synthesized on free polysomes and released into the cytosol as soluble species. Most of these exist as large precursors containing amino-terminal targeting preseqences (Hartl *et al.*, 1989). Precursor proteins must be made import competent by conformational alterations that occur in an ATP-dependent reaction that may involve the action of heat shock proteins (Pelham, 1986; 1988). It is well known that certain families of heat shock proteins in periods of stress bind to hydrophobic regions of denatured polypeptides and prevent aggregation in an ATP-dependent manner (Pelham, 1984; Lewis and Pelham, 1985). The heat shock protein, designated hsp60, in yeast and *N.crassa* has been implicated as a being a member of a subclass of proteins known as "chaperonins", which ensure proper folding and assembly of some mitochondrial proteins into multimeric structures

(Hemmingsen *et al.*, 1988; Ostermann *et al.*, 1989; Reading *et al.*, 1989).

Precursor proteins then recognize proteinaceous import receptors on the surface of the outer membrane (Zwizinski *et al.*, 1983; Hartl *et al.*, 1989). Following receptor binding, precursor polypeptides are transferred to an insertion site in the outer membrane, called the general insertion protein (GIP) (Pfanner *et al.*, 1988a; Pfaller *et al.*, 1989). The large precursor molecules, except for those destined to the outer membrane, are then transported across the membrane(s) and sorted to the correct mitochondrial compartment (Hartl *et al.*, 1989). Translocation into or across the inner membrane occurs at contact sites, where the outer and inner membranes touch. This translocation step is dependent on a membrane potential across the inner membrane (Schwaiger *et al.*, 1987; Hartl *et al.*, 1989) which is thought to drive electrophoretically the positively charged regions of the precursor protein into or across the inner membrane (Roise *et al.*, 1986).

Precursor proteins are then processed by a metal-ion dependent processing peptidase (MPP), located in the matrix (Hawlitschek *et al.*, 1988; Hartl *et al.*, 1989). Correct sorting of proteins destined for the intermembrane space occurs via a second hydrophobic presequence which directs these proteins back into the inner membrane (Gasser *et al.*, 1982b; Pratje *et al.*, 1983; Pratje and Guiard, 1986; Hartl *et al.*, 1987). For such proteins a second proteolytic cleavage may occur on the outer face of the inner membrane (Gasser *et al.*, 1982; Bohni *et al.*, 1983).

After localization to the correct mitochondrial compartment, the protein may undergo further post-translational modification (Hartl *et al.*, 1989). The additional processing steps include covalent and non-covalent modifications such as heme attachment, iron-sulfur cluster formation and conformational changes (Hartl *et al.*, 1989). Many proteins are further assembled into large protein complexes (Hartl *et al.*, 1989).

The import of cytochrome *c* into mitochondria in *N.crassa* (see Figure 3) does not occur via the standard mechanisms outlined above. Cytochrome *c* is translated as a precursor, apocytochrome *c*, which lacks an N-terminal presequence (Zimmermann *et al.*, 1981). It is not proteolytically processed upon import, and is targeted directly to the intermembrane space (Zimmermann *et al.*, 1981; Stuart *et al.*, 1987; Hartl *et al.*, 1989; Stuart, 1989).

Recently, it has been determined that the import of cytochrome *c* is not dependent upon NTPs, in contrast to most proteins targeted to mitochondria (Stuart, 1989; Stuart *et al.*, 1990). This suggests that apocytochrome *c* requires a certain folded structure to be maximally competent for import and unfolding the apo-protein makes it less competent for import.

Apocytochrome *c* can spontaneously insert into the lipid domains of the outer membrane (Berkhout *et al.*, 1987; Mannella *et al.*, 1987), where it binds with high affinity to a specific receptor (Hennig and Neupert, 1981; Hennig *et al.*, 1983). This receptor is thought to exist as a complex which includes cytochrome *c* heme lyase, the enzyme that attaches heme to apocytochrome *c* (Nicholson *et al.*, 1988; Nicholson and Neupert, 1988). It has been postulated that cytochrome *c* heme lyase might act as a specific binding site *in lieu* of a surface receptor (Nicholson *et al.*, 1988; Stuart, 1989), since apocytochrome *c* import is blocked at the stage of high-affinity receptor binding if heme attachment is inhibited by the analogue deuterohemin, and is reversed by adding protohemin (Hennig and Neupert, 1981). This hypothesis is consistent with the observation that the mitochondria of the *N.crassa cyt-2-1* mutant, which lacks CCHL activity, are also deficient in their ability to bind apocytochrome *c* (Nargang *et al.*, 1988; Stuart, 1989).

Since bound apocytochrome *c* is sensitive to proteinase until holocytochrome *c* is formed, it appears that the formation of holocytochrome *c* is a prerequisite for cytochrome *c* import (Hennig and Neupert, 1983; Nicholson *et al.*, 1988; Nicholson and Neupert, 1989; Stuart, 1989). The attachment of the heme group to the partially inserted apocytochrome *c* is thought to induce a conformational change which is responsible for driving the translocation of cytochrome *c* across the outer membrane into the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*, 1987b; Hartl *et al.*, 1989; Stuart, 1989).

Currently it is thought that the ability of apocytochrome *c* to spontaneously insert into the outer membrane and the fact that enough free energy is generated by the refolding of cytochrome *c* after heme attachment to drive holocytochrome *c* across the outer membrane (Nicholson *et al.*, 1987a), allows for the circumvention of the general import pathway (Stuart, 1989; Stuart *et al.*, 1990).

The Present Study

The studies undertaken in this thesis were originally designed to understand and characterize the nature of the cytochrome *c* deficient mutant *cyc-1-1*. A *N.crassa* genomic cosmid library was screened by sib selection for its ability to rescue the slow growth character of *cyc-1-1*. It was thought that DNA sequence analysis of the wild-type gene capable of rescuing *cyc-1-1* might give insight into some of the complex interactions important in the regulation of cytochrome production in *N.crassa*.

However, during the course of this study it became apparent that the *cyc-1-1* mutant might be related to another *N.crassa* mutant, namely *cyt-12-1*. Therefore, experiments were undertaken to investigate this possiblity. These studies showed that *cyc-1-1* and *cyt-12-1* were allelic mutations. Because of these experiments and because a publication on the *cyc-1-1* mutant appeared during the course of the work (Stuart *et al.*, 1987), the project was altered to investigate the nature of the *cyt-12-1* mutant.

II. Materials and Methods

Media and Buffers

The composition of all media, and certain complex buffers, and solutions are given in the appendix. The composition of all other buffers and solutions are given in the text of the appropriate section.

E.coli Strains and Culture Conditions

The *E.coli* strains used are described in Table 1. The bacterial strains were grown at 37°C in liquid L-broth medium (Lennox, 1955; see appendix) with shaking (200 rpm) or on L-broth plates. Overnight cultures of JM103 were grown in DM salts (Davis and Mingioli, 1950) containing 10 mM thiamine and 0.5% (w/v) glucose (see appendix). Antibiotics, for selective purposes, were added to the autoclaved medium after it had cooled to approximately 55 to 60°C. Filter-sterilized tetracycline·HCl or ampicillin were added to the medium to a final concentration of 20 µg/ml or 100 µg/ml, respectively. When screening bacterial strains harboring pUC19 or M13 phage derivatives for DNA fragment insertions, X-GAL (dissolved in N,N-dimethyl-formamide) and IPTG (dissolved in distilled water) were added to a final concentration of 50 µg/ml and 25 µg/ml, respectively.

Glycerol stocks of the *E.coli* strains, were prepared as described in Maniatis *et al.* (1982); 850 µl from a saturated overnight culture was added to 150 µl sterile glycerol in a sterile microcentrifuge tube. After gentle mixing, these glycerol stocks were stored at -20°C.

Neurospora crassa **Strains and Culture Conditions**

The strains of *N.crassa* that were used in this study are described in Table 2. Conidial growth of *Neurospora* was in 250 ml Erlenmeyer flasks containing modified Vogel's medium (Davis and de Serres, 1970; see appendix) and 1.5% (w/v) agar. The flasks were incubated at 30°C until adequate mycelial growth was observed. At this time the flasks were taken out of the incubator and exposed to light at room temperature. After conidiation, the conidia were suspended in sterile distilled water. The suspension was filtered through two layers of sterile cheesecloth in a sterile Buchner funnel to remove the fragments of mycelium. The conidial concentration was determined by using a haemocytometer. Conidia were used as inoculum for liquid Vogel's medium to yield a final concentration of 1 to 3 X 10^6 conidia/ml. The culture was incubated at 30°C or 37°C with vigorous shaking until it reached the appropriate stage of growth (usually mid-log phase).

Preservation of ***Neurospora*** **Strains**

Neurospora strains were kept on silica gel as previously described for long-term preservation (Davis and de Serres, 1970). A dram screw-cap vial half-filled with silica gel (grade H, type II, 6-12 mesh) was heated at 180°C for 90 minutes, with the caps loosened to activate the silica gel. At this time, the cap was tightened and the vial was allowed to cool to room temperature. A sterilized solution of 10% (w/v) reconstituted skim milk (1 to 2 ml) was used to suspend conidia from fresh Vogel's slants (< 10 days old) with a sterile pasteur pipet. Enough suspension was added to the sterile dram vial to be fully absorbed by the activated silica gel. The sealed vial was vortexed vigorously and quickly placed in an ice-water bath for 10 minutes. After one week, if the silica was not dry or growth was apparent at room temperature, the vial was discarded. One or two of the silica stock crystals were used to inoculate solid Vogel's medium for conidial growth.

Heterokaryon Construction

Heterokaryons were made by superimposing two different auxotrophic *N.crassa* strains of the same mating type onto unsupplemented Vogel's medium (Bertrand and Pittenger, 1969).

Cytochrome Spectra

Cytochrome spectra were obtained by a modification of the procedure described by Bertrand and Pittenger (1969) using a Shimadzu UV-265 recording spectrophotometer. A crude mitochondrial pellet was resuspended in 1.5 ml of

TE buffer (10 mM Tris·HCl, pH 7.8, 1 mM EDTA). The mitochondria were dissolved by adding 1.5 ml of 5% (w/v) sodium deoxycholate, 10 mM Tris·HCl, pH 7.8, 10 mM EDTA. The mixture was transferred to two microcentrifuge tubes, the tubes were centrifuged for 2 minutes, and the supernatant was decanted to a test tube. The sample was mixed thoroughly with a pasteur pipet before determining the spectrum. The air-oxidized mitochondrial solution was used to generate a baseline by scanning from 650 nm to 500 nm. The solution in the reference cuvette was completely oxidized with a few crystals of potassium ferricyanide. The solution in the sample cuvette was reduced by adding a few grains of sodium dithionite. The spectrophotometric scan was repeated. The primary absorption peaks for cytochrome *c*, cytochrome *b* and cytochrome aa_3 are at 550 nm, 560 nm and 608 nm, respectively (Bertrand and Pittenger, 1972).

Plasmid and Cosmid DNA Vectors

Plasmids pBR322 (Bolivar *et al.*, 1977), pUC19 (Yanisch-Perron *et al.*, 1985) and the cosmid vector pSV50 (Vollmer and Yanofsky, 1986) were used as cloning vectors. (Table 3.) Table 4 lists the recombinant plasmids constructed or obtained from others for use in this study.

Subcloning

Purified DNA fragments were cut with various restriction endonucleases and ligated into the compatible cloning sites of the M13 vectors mp18 and mp19 (Messing, 1983) and the plasmid vector pUC19 (Yanisch-Perron *et al.*, 1985). The M13 and pUC19 ligation products were then transfected or transformed into competent JM103 and JM83 cells, respectively.

Transformation

A large sterile morton capped test tube, containing 10 mls of L-broth, was inoculated with the appropriate *E.coli* strain. After growing the culture overnight to saturation at 37°C with vigorous shaking, 200 µls of the overnight culture was used to inoculate a sterile 250 ml Erlenmeyer flask, containing 40 mls of L-broth. The culture was grown at 37°C in a shaker-incubator until the culture reached mid-log phase. The bacterial cells were pelleted by centrifugation (5,000 rpm for 4 minutes)[1]. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile 30 mM $CaCl_2$. After incubating on ice for 30 to 90 minutes, 200 µl of competent cells were transferred to a sterile microcentrifuge tube per transformation. After the addition of the ligation mixture to the competent cells, the suspension was incubated on ice for 30 to 90 minutes. The transformation mixture was then heat-shocked at 42°C for 2 minutes before adding 500 µl of sterile L-broth. After mixing by inversion, the tube was incubated at 37°C for 30 minutes. Aliquots of 25 to 150 µl were plated on selective media and incubated at 37°C overnight.

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[1] Unless otherwise stated, centrifugation was in a Sorvall (SS-34 rotor) at 4°C.

M13 Transfection

M13 single-stranded or M13 replicative form DNA was transfected into JM103 as previously described by Norgard *et al.*(1978). After the addition of DNA to the *E. coli* competent cells and the 30 minute incubation on ice, the transfection mixture was heat-shocked at 42°C for 2 minutes. Different amounts of the transfection mixture were added to small sterile morton closure capped tubes containing: 3 ml of soft agar (see appendix) supplemented with 200 µl of a saturated JM103 overnight culture, 50 µl of X-GAL solution and 10 µl of IPTG solution. This mixture was overlayed onto L-broth plates and incubated at 37°C for at least 9 hours.

Rapid Plasmid DNA Isolation

Mini-plasmid DNA preparations were isolated by a slight modification of the alkaline-lysis protocol of Birnboim and Doly (1979). The plasmid-containing *E.coli* strain was grown overnight in 10 mls of L-broth plus the appropriate antibiotic. The cells were pelleted (5,000 rpm for 5 minutes) and the supernatant discarded. The pellet was resuspended in 200 µl of cold glucose buffer (50 mM glucose, 25 mM Tris·HCl, pH 7.5[2], 10 mM EDTA). The cell suspension was placed in a microcentrifuge tube containing 400 µl of freshly prepared alkaline-SDS (0.2 M NaOH, 1% (w/v) SDS). After the tube was mixed by inversion, it was left on ice for 5 minutes. The proteinaceous material and non-supercoiled DNA was precipitated by the addition of 300 µl of cold 3 M sodium acetate, pH 4.8 and gentle mixing by inversion. The tube was centrifuged in a microcentrifuge for 5 minutes.[3] The supernatant (approximately 750 µl) was added to a new tube, containing 450 µl of cold isopropanol, mixed thoroughly and left at -20°C for 5 minutes. The DNA was pelleted by centrifugation for 5 minutes. The supernatant was removed by aspiration and the pellet was dried in a vacuum dessicator. The pellet was resuspended in 200 µl of distilled water. The DNA was reprecipitated by adding 150 µl of cold 3 M sodium acetate, and filling the tube with 95% ethanol. After mixing thoroughly by inversion, the tube was centrifuged for 5 minutes. The supernatant was removed by aspiration and the nucleic acid pellet was with 95% ethanol. After mixing thoroughly by inversion, the tube was centrifuged for 5 minutes. The supernatant was removed by aspiration and the nucleic acid pellet was dried in a vacuum dessicator. The pellet was resuspended in 100 µl of distilled water and 10 µl was used in typical 50 µl restriction digests. During digestion 2 µl of RNase A (10 mg/ml stock solution; Maniatis *et al.*, 1982) was added to degrade the RNA. The rapid-plasmid DNA isolated by this method did not produce reasonable transformation frequencies in *Neurospora* transformation experiments but was useful for screening recombinant pUC19 clones for DNA fragment insertions..

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[2] The pH of the buffers containing Tris was adjusted at room temperature.

[3] Unless otherwise stated, centrifugation in the microcentrifuge was done at room temperature.

Large-Scale Plasmid and Cosmid DNA Isolations

Plasmid DNA was isolated by a modification of the "alternate" procedure given in Maniatis *et al.*(1982). An overnight culture (1 ml) of the plasmid-harboring *E.coli* strain was used to inoculate 1 l of L-broth. The culture was grown overnight (approximately 13 to 18 hours) in a 37°C shaker-incubator (200 rpm), until saturation was achieved. The cells were pelleted by centrifugation in a Sorvall GSA rotor (3,000 rpm for 5 minutes at 4°C). The supernatant was discarded and the pellets were suspended in approximately 50 ml of STE buffer (150 mM NaCl 50 mM Tris·HCl, pH 7.8, 1 mM EDTA). The cells were repelleted by centrifugation as stated above. The supernatant was decanted and the pellets were resuspended in a combined volume (20 ml) of sucrose buffer (10% (w/v) sucrose, 50 mM Tris·HCl, pH 8.0). To this mixture, 16 ml of 0.25 M EDTA, pH 8.0 and 4 ml of a freshly prepared solution of lysozyme (10 mg/ml in 0.25 M Tris·HCl, pH 8.0) was added. The solution was mixed by inversion and left on ice for 30 minutes. To this solution 8 ml of 10% (w/v) SDS was added, mixed quickly and 12 ml of 5 M NaCl was added. The suspension was then mixed gently by inversion and left in an ice-water bath for 1 hour. The viscous solution was transferred to polycarbonate tubes and centrifuged (18,000 rpm for 30 minutes) to remove the high molecular weight DNA. The supernatant was decanted to fresh SS-34 tubes and 0.6 volumes of cold isopropanol was added. The solution was mixed well and left on ice for 10 minutes. The nucleic acids were pelleted by centrifugation (12,000 rpm for 10 minutes). The supernatant was discarded and the pellets were dried in a vacuum dessicator. The pellets were resuspended in a total volume of 4 ml of distilled water with 5 ml pipets. The insoluble material was pelleted by centifugation (10,000 rpm for 5 minutes). The supernatant was decanted to a fresh SS-34 tube, 4 g of cesium chloride was added and dissolved by inversion. Ethidium bromide (750 µl of a 10 mg/ml solution) was added and the mixed solution was centrifuged (15,000 rpm for 25 minutes). The supernatant was transferred to 13 x 51 mm Quick-Seal^{TM} tubes (Beckman). The plasmid DNA was banded by equilibrium-density centrifugation in a VTi65 rotor

(Beckman) at 54,000 rpm for 6 hours at 20°C. Plasmid DNA collected from the gradients was extracted four times with salt-saturated isopropanol (see appendix) to remove the ethidium bromide from the aqueous solutions. The cesium chloride concentration was reduced by dialysis against 5 l of distilled water for 1 hour to overnight at 4°C. The DNA solution was transferred to microcentrifuge tubes and 20 µl of RNase A (10 mg/ml stock) was added. After mixing by inversion and incubating for 30 minutes at 37°C, 20 µl of Protease K (see appendix) was added. The solution was left for 30 minutes at 37°C. The DNA solution was extracted four times with water-saturated phenol and once with chloroform/iso-amyl alcohol (see appendix). The DNA solution (approximately 500 µl or less) was ethanol precipitated by adding 150 µl of 3 M sodium acetate, pH 4.8 and filling the tube with 95% ethanol. The nucleic acids were pelleted in a microcentrifuge for 20 minutes. The pellet was washed once with 70% ethanol and dried in a vacuum dessicator. The DNA pellet was resuspended in 300 µl of sterile water. A small aliquot was diluted to determine the DNA concentration using a spectrophotometer. Restriction digests, using appropriate restriction endonucleases, were electrophoresed on 0.8% agarose gels to confirm that the plasmid isolated was correct.

Isolation of M13 Single-Stranded DNA

M13 single-stranded DNA was isolated by a modification of the procedure found in "M13 Cloning and Sequencing System: A Laboratory Manual", New England Biolabs, Inc. Sterile pasteur pipets were used to pick individual plaques and blown into separate 250 ml Erlenmeyer flasks, that contained 40 ml of L-broth inoculated with 150 µl of cells from a fresh JM103 overnight culture. The culture was grown in a shaker-incubator with vigorous shaking at 37°C for 9 to 11 hours. The cells were harvested by centrifugation (15,000 rpm for 30 minutes). The supernatant was decanted to a clean SS-34 tube and 7 ml of 10% (w/v) PEG-8000, 2.5 M NaCl was added and the tubes were mixed by inversion. After the mixture had been on ice for 1 hour, the precipitated phage were pelleted by centrifugation (10,000 rpm for 20 minutes). The pellet was suspended in 650 µl of phenol extraction buffer (0.3 M NaCl, 0.1 M Tris·HCl, pH 7.3, 1 mM EDTA) and transferred to a microcentrifuge tube. To this solution 10 µl of Protease K solution (see appendix) and, 10 µl of a 10% (w/v) SDS solution were added and incubated at 37°C for 30 minutes. This solution was extracted with water-saturated phenol four times and once with chloroform/iso-amyl alcohol (see appendix). The single-stranded phage DNA was precipitated by filling the tube with 95% ethanol. After mixing by inversion, the DNA was pelleted by centrifugation in a microcentrifuge for 5 minutes and washed once with 70%

ethanol. The DNA pellet was dried in a vacuum dessicator and resuspended in 100 µl of distilled water. The single-stranded clones to be used in sequencing reactions were screened by running appropriate single-stranded DNA standards, whose nucleic acid concentration and size were known, adjacent to the clones, on a 0.8% agarose gel. The amount of the clone used for DNA sequencing was estimated by comparing the florescence of the clone to the standards on a UV-light trans-illuminator.

Single Stranded M13 Subcloning

In certain cases the Dale method was used to obtain DNA sequence of the wild-type cytochrome *c* gene (Dale *et al.*, 1985). The wild-type cytochrome *c* gene was cloned into M13mp19 and the single-stranded DNA obtained was used to generate deletions as follows. To a clean microcentrifuge tube single-stranded DNA (2 to 4 µg), 2 µl of 10X EcoRI restriction buffer (BRL),and 2 µl (10 ng)of RD20 Dale primer were added. The volume was made up to 18 µl with sterile distilled water. The primer was annealed to the template by placing the tube at 65°C for 2 minutes and allowed to cool slowly to 40°C. The double stranded region was then cut by adding 2 µl of EcoRI (10 units/µl) restriction endonuclease and incubating at 42°C for 1 hour. A small aliquot (3 µl) was removed and electrophoresed adjacent to an aliquot of uncut single-stranded template on a 0.8% agarose gel to determine if digestion was complete.

Uni-directional deletions were made by adding the following to the remaining 17 µl solution: 1 µl of BSA (4 mg/ml), 1 µl of 0.2 M dithiothreitol, 1 µl of 1.0 M Tris·HCl, pH 7.5 and 1 to 2 units of T4 DNA polymerase (Pharmacia). During the 37°C incubation, 5 µl aliquots were removed every 15 minutes and transferred to a single microcentrifuge tube on ice. The T4 DNA polymerase was heat inactivated by placing the tube at 65°C for 10 minutes. A poly-G tail was added to the end of the deletions by adding the following: 2.5 µl dGTP (50 mM), 2.0 µl water and 0.5 µl of terminal deoxy-transferase (BRL, 8 units/µl). The reaction was incubated at 37°C for 20 minutes and stopped by a 10 minute incubation at 65°C. To the above reaction 2 µl of Dale primer was added and annealed to the template as before. The following ligation was prepared: 12 µl of the Dale reaction, 1.5 µl of ATP (10 mM), 1.5 µl of water and 1 µl of T4 DNA ligase (6 units/µl). After incubation at room temperature for 4 hours, 1 to 5 µl of sample was used for *E.coli* transfection.

Preparation of *Neurospora* Sphaeroplasts

The preparation of frozen *Neurospora* sphaeroplasts essentially followed the procedure described by Akins and Lambowitz (1985). Fresh conidia (<2 weeks old) from the *Neurospora* strain to be transformed were used to prepare a conidial suspension in sterile water. The concentration of this suspension was determined by using a haemocytometer.

Enough conidial suspension to yield a final concentration of 5 X 10^6 to 1.5 X 10^7 conidia/ml was added to a 2 l baffled Erlenmeyer flask containing 1 l of 0.5X liquid Vogel's medium (see appendix). The culture was incubated with gentle shaking (200 rpm) at 25 to 30°C. The extent of germination was observed by counting the number of germinated conidia as a percentage of the total number of conidia in a haemocytometer. A conidium was considered germinated when a small protuberance was observed. Once germination (>80%) was achieved the conidia were harvested by centrifugation (5,000 rpm for 10 minutes) in sterile GS-3 rotor bottles. The pelleted conidia were washed twice with sterile distilled water and twice with sterile 1 M sorbitol. A small aliquot was removed by a sterile pasteur pipet, to determine the concentration of the conidial suspension using a haemocytometer. The conidial pellet was resuspended in 9 ml of sterile 1 M sorbitol. The volume was adjusted, with additional 1M sorbitol, such that the maximum concentration was 1 X 10^9 conidia/ml. The suspension was transferred to a sterile 250 ml Erlenmeyer flask, 15 mg of Novozym™ 234 (Novo Laboratories, Inc.) was added for each ml of suspension and incubated at 30°C with gentle shaking (100 rpm) for 1 hour. The percentage of sphaeroplasting was monitored microscopically by comparing the ratio of "ghosts" (lysed conidia) in a SDS-treated sample to the number of conidia in an untreated sample. After 90% sphaeroplasting was achieved, the sphaeroplasts were pelleted by centrifugation in a IEC clinical centrifuge (10 minutes at low speed). The pellets were washed twice with sterile 1 M sorbitol and once with sterile MCS solution (20 mM MOPS, pH 6.3, 50 mM $CaCl_2$,1 M sorbitol). The sphaeroplasts were resuspended in 6 ml of sterile MCS. The volume was adjusted with additional sterile MCS such that the sphaeroplast concentration was between 2.5 to 5 X 10^8 sphaeroplasts/ml. The total volume of the solution was measured and for each ml of solution the following was added: 13 µl dimethylsulfoxide, 65 µl of a sterile heparin solution (5 mg/ml) and 275 µl of PMC solution (see appendix). The mixture was gently mixed by inversion and the sphaeroplasts were aliquoted into sterile micro-centrifuge tubes and stored at -70°C. At a later date, a tube of sphaeroplasts was thawed on ice and a dilution series using sterile 1 M sorbitol was prepared. The sphaeroplast viability was determined by plating 100 µl of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions onto viability plates (see appendix) using top agar (see appendix). If a viability of greater than 10^7/ml was not obtained the sphaeroplasting procedure was repeated.

Transformation of *Neurospora* Sphaeroplasts and Isolation of *Neurospora* Genes by Sib Selection

These procedures were as described by Akins and Lambowitz (1985) utilizing a wild-type (*74A*) *Neurospora* genomic library constructed in the pSV50 cosmid vector (Vollmer and Yanofsky, 1986). Frozen sphaeroplasts were thawed on ice. Approximately 5.0 µg of DNA per transformatiom was used in the first two rounds of sib-selection while later rounds required less DNA because of the decreasing complexity. In all cases the sphaeroplast to DNA ratio was kept in the range of 6 X 10^6 to 3.5 X 10^7 viable sphaeroplasts/µg of DNA. To a sterile micro-centrifuge tube, DNA was added with sterile distilled water such that the total volume was 60% of the sphaeroplast volume. The sphaeroplasts were added and the tubes were incubated on ice for 30 minutes after gently mixing by inversion. To the microcentrifuge tube, sterile PMC solution (10X the sphaeroplast volume; see appendix) was added, gently mixed and incubated at room temperature for 20 minutes. To a sterile 125 ml Erlenmeyer flask, top agar (10 ml/plate; see appendix) and the transformation mixture was added and gently shaken. The contents of the flask were overlayed onto benomyl plates (see appendix) and incubated at 37°C.

Maintenance of the Cosmid Library

The wild-type (*74A*) *Neurospora* library screened in this study comprises of 3168 separate *E.coli* clones stored in microtiter dishes at -70°C. The actual maintenance and the utilization of the genomic library is as described by Akin and Lambowitz (1985) and Drygas *et al.* (1989).

Isolation of *N.crassa* Transformants for Spectra

Cyt-12-1 sphaeroplasts were transformed with cosmids (pSV50-5324 and pSV50-4333) and plasmids (pSP8 and pDB1) as described in the transformation of *Neurospora* sphaeroplasts and isolation of *Neurospora* genes by sib selection section above. Fast growing colonies were picked using a sterile pasteur pipet and blown into separate sterile 250ml Erlenmeyer flasks containing 40 ml of Vogel's medium, 1.5% (w/v) agar (see appendix), and 0.5 mg/l benomyl (dissolved in 95% ethanol). After incubating at 30°C, until adequate mycelial growth was observed, the flasks were removed from the incubator and placed in the light. When sufficient conidial growth was apparent, the conidia were harvested with sterile distilled water and used as inoculum for liquid Vogel's medium containing 0.5 mg/ml benomyl and growed at 30°C in a shaker-incubator (200 rpm). When the cultures reached mid-log phase, they were harvested by filtration Cytochrome spectra of the transformants followed the procedure previously described in the cytochrome spectra section.

DNA Sequencing

DNA sequencing of the wild-type cytochrome *c* gene followed the dideoxy method of Sanger *et al.* (1977) using M13 vectors (Messing, 1983). Single-stranded DNA clones were constructed using M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985). The hybridizations for sequencing contained the following: 1.0 to 2.0 µg of the single-stranded DNA template, 10 ng of the appropriate primer from 16 to 20 base pairs long (One of: M13-17mer@-20, M13-17mer@-40, New England Biolabs, Inc.; cytc1, cytc2, cytc3, cytc4, (see Fig.17) Dr. Ken Roy, Department of Microbiology), and 1 µl of 10X Pol buffer (see appendix). The total volume was made up to 12 µl with sterile water in a microcentrifuge tube. To anneal the primer and template, the tube was placed in a boiling waterbath for 3 minutes and then cooled for 3 minutes at room temperature. The hybridization mixture was transferred to a well of a Nunc Microwell plate™.and 1 µl of 0.1 M dithiothreitol , 1 µl of radioisotope (α^{32}P-dATP (>600 Ci/mmol; NEN) or α^{35}S-dATP (>1000 Ci/mmol; Amersham) and 1 µl of Klenow fragment (1 unit/µl) were added. After mixing thoroughly, 3 µl aliquots were dispensed to four labelled adjacent wells of the microtiter dish. The elongation reactions were initiated by adding 3 µl of the A, C, G or T mixes (see appendix) to the appropriate wells and incubating in an air-incubator (42 to 55°C). When secondary structures were encountered sequencing was repeated using, either c^{7}dGTP (Barr *et al.*, 1986; see appendix) or dITP (Sanger *et al.*, 1982; see appendix) in place of dGTP in all of the reaction mixes. After incubating 10 minutes or 15 minutes (depending if α^{32}P-dATP or α^{35}S-dATP was used, respectively), 1.5 µl of chase (see appendix) was added to each of the four reactions and the plate was returned to the incubator for 10 minutes. The sequencing reactions were stopped by adding 14 µl of dye-formamide mix (see appendix) to each reaction. The samples were transferred to labelled microcentrifuge tubes and the template-radioactive strands were denatured by boiling for 5 minutes.

The *cyt-12-1* mutant gene was sequenced by the T7 polymerase dideoxy sequencing method (T7 Sequencing™ Kit, Pharmacia LKB Biotechnology, 1988) using the radioisotope α^{35}S-dATP. When areas of sequence compression were observed the sequence was redone and dGTP was replaced in all reaction mixes with c^7dGTP or dITP (Deaza T7 Sequencing™ Mixes, Pharmacia LKB Biotechnology, 1988).

The reactions (3 µl) were loaded onto 6% acrylamide sequencing gels (prepared from a 40% acrylamide stock (see appendix), 8.33 M urea, 0.1 M Tris·borate, pH 8.3, 2 mM EDTA). The short gels (40 cm X 20 cm X 0.25 mm) and long gels (80 cm X 20 cm X 0.25 mm) were run at 27 and 50 Watts (constant power), respectively. When α^{35}S-dATP was the radioisotope used, the acrylamide gels were dried in a Bio-Rad Slab Dryer (Model 483) at 80°C for 2 hours. Autoradiography of dried gels was performed at room temperature using XAR-5 X-ray film (Kodak) for 12 to 72 hours, depending upon band intensities, before being developed according to the manufacturer's instructions. Most ^{32}P labelled gels were not dried; autoradiography was achieved at -20°C or -70°C and the X-ray film was developed after 12 to 36 hours.

Agarose Gel Electrophoresis

Gels were made to 0.8% (w/v) agarose in TAE buffer (0.04 M Tris·acetate, 1 mM EDTA) and contained 0.5 µg/ml ethidium bromide. 1/10 volume of loading dye (50% (v/v) glycerol, 0.25% (w/v) bromophenol, 0.25% (w/v) xylene cyanol) was added to the DNA samples just prior to loading. Electrophoresis was performed at 25 to 150 Volts by a constant voltage power unit (BRL).

Purification of DNA from Agarose Gels

DNA was isolated from agarose gels by the electro-elution procedure described by Maniatis *et al.* (1982). After electrophoresis, the portion of the agarose gel containing the DNA band of interest was cut out. The slice was placed in a dialysis bag containing TAE buffer and the ends sealed with dialysis clips. The DNA was electro-eluted from the gel slice onto the inner wall of the dialysis bag by placing in a gel apparatus containing TAE buffer and applying 100 volts (constant voltage) for 30 to 90 minutes. The polarity was reversed for 2 minutes to release the DNA from the walls of the bag. The dialysis bag was removed and the aqueous solution was recovered by using a pasteur pipet and transferred to microcentrifuge tubes. The DNA solution was extracted four times with water-saturated phenol and once with chloroform/iso-amyl alcohol. The DNA was precipitated by adding 150 µl of 3 M sodium acetate, pH 4.8 and filling the tubes with 95% ethanol. The tubes were centrifuged for 20 minutes and the pellets were washed once with 70% ethanol. The pellets were dried in a vacuum dessicator and resuspended in a total volume of 100 µl of sterile distilled water.

Isolation of *Neurospora* DNA

Cyt-12-1 nuclear DNA was isolated by minor modifications of the procedure described by Schechtman (1986). Fresh conidia (< 10 days old) were used as inoculum for liquid Vogel's medium (1 l) in sterile 2 l baffled Erlenmeyer flasks to yield a final concentration of 10^6 conidia/ml. The cultures were grown at 30°C in a shaker-incubator until they reached mid-log phase, then the mycelium was harvested by filtration and washed once with cold distilled water. The mycelial pad was transferred to a baked mortar containing liquid nitrogen. Using a pestle the mycelium was ground to a very fine powder. The powder was then transferred evenly among three baked 30 ml corex tubes before adding 15 ml of 50 mM EDTA, pH 8.5, 0.2% (w/v) SDS and 15 µl of DEPC to each tube. The tubes were vigorously shaken to dislodge the frozen material, then gently mixed by inversion for 1 minute. The mixture was incubated at 70°C for 15 minutes prior to placing on ice for at least 10 minutes. After the incubation, 950 µl of

8M potassium acetate, pH 4.3 was added. The solution was mixed by inversion, then placed on ice for 1 hour. The cellular material was pelleted by centrifugation (9,000 rpm for 25 minutes at 4°C). The supernatant was transferred to fresh corex tubes. The DNA was precipitated by adding an equal volume of cold isopropanol and gently mixing by inversion. The DNA clot was washed once with 70% ethanol, drained, and air dried in a vacuum dessicator. The pellet was resuspended in 8 ml of 1 mM EDTA, pH 8.0. To this solution 4 ml of high salt buffer (100 mM NaCl, 20 mM Tris·HCl, pH 7.8, 2 mM EDTA, pH 8.0) and 30 µl of pre-digested RNase A (10 mg/ml) was added. After incubating for 30 minutes at 37°C, 180 µl of spermine·HCl, pH 7.0 was added, gently mixed by inversion, and the supernatant was discarded. The pellet was washed 3 times for minutes each, while on ice, with 2 ml of cold spermine wash buffer (75% ethanol, 10 mM magnesium aceate, 300 mM sodium acetate, pH 6.0) pipetting off the rinse each time. The DNA pellet was rinsed once with 70% ethanol before air dried in a vacuum dessicator. The pellet was resuspended in 2 ml of 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl by placing on an aliquot mixer overnight at 4°C. The DNA was reprecipitated by adding 4 ml of 95% ethanol. The supernatant was removed by using a pipet and the pellet was washed once with 70% ethanol prior to air drying in a vacuum dessicator. The DNA pellet was suspended in a final volume of 1 ml of TE buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA). A small aliquot was removed to determine the DNA concentration using a spectrophotometer before restriction endonuclease digestion.

DNA Isolation Using Continuous Sucrose Gradients

To isolate the *cyt-12-1* mutant gene, large-scale preparation of sized genomic DNA was performed as described by Maniatis *et al.* (1982) . The sucrose solutions (19 ml of 10% (w/v) and 19 ml of 40% (w/v)) were made in sucrose buffer (1 M NaCl, 20 mM Tris·HCl, pH 7.8, 5 mM EDTA). The sucrose gradient was prepared in a 38 ml SW27 (Beckman) polyallomer tube by using a gradient mixer (Tyler Instruments, Inc.). Genomic DNA (250 to 500 µg) was digested with restriction enzymes, HindIII and PstI. The DNA solution was carefully loaded to the top of the sucrose gradient. The tubes were centrifuged at 26,000 rpm for 24 hours at 20°C in a SW28 rotor (Beckman, Inc.). After spinning, the bottom of the centrifuge tube was carefully punctured using a syringe needle and 500 µl fractions were collected in labelled sterile microcentrifuge tubes. A 10 µl aliquot of every odd fraction was transferred to a fresh micro-centrifuge tube. To each sample, 10 µl of water and 5 µl of loading dye (see appendix) was added. After mixing thoroughly, the solutions were loaded on a 0.8% (w/v) agarose gel and electrophoresed overnight at 50 volts (constant voltage). Following electrophoresis, the DNA was observed by using a UV-light trans-illuminator. Only those fractions that contained DNA in the size range (2 to 5 kb) were pooled and precipitated by adding 2 volumes of 95% ethanol. The DNA was pelleted by centrifugation in a microcentrifuge. The pellet was dried in a vacuum dessicator and resuspended in 200 µl of TE buffer (10 mM Tris·HCl, pH 7.8, 1 mM EDTA). A 10 µl sample was removed, 10 µl of water and 2 µl of loading dye was added. The DNA solution was loaded on a 0.8% (w/v) agarose gel to estimate concentration and molecular weight prior to cloning into pUC19.

RNA Isolation

Most *Neurospora* total RNA was isolated by minor modifications to the phenol-SDS procedure described by Reinert *et al.* (1981). All glassware and most solutions used with RNA were DEPC-treated prior to baking and autoclaving, respectively. *Neurospora* cultures were grown in liquid Vogel's medium at 30°C until mid-log phase was achieved. The mycelium was quickly harvested by filtration and washed three times with ice-water. The weight of the mycelial pad was recorded after taking a portion for cytochrome spectral analysis. The pad was quickly frozen in liquid nitrogen. Occasionally the pads were stored at -70°C for a few days before being processed. While still frozen, 1.5 g of baked, acid washed, sea sand was added for each gram of mycelium. The mycelium was ground to a fine powder and left to thaw slightly. For every gram of mycelium, 3 ml of part A extraction buffer (see appendix) was added. Quickly a smooth paste was made and transferred to 30 ml corex tubes as described above. Immediately an equal volume of part B extraction buffer (see appendix) was added to each tube and mixed thoroughly for 30 minutes by placing them on an aliquot mixer. The tubes were centrifuged in a SS-34 rotor at 10,000 rpm for 15 minutes at room temperature. The phenol phase of each tube was transferred to clean corex tubes placed on ice. The aqueous phase and interphase of each tube was re-extracted with 2 ml of part A extraction buffer. The solutions were mixed and centrifuged as before. The aqueous phase from the re-extraction was added to the tubes on ice. The nucleic acids were precipitated by adding 2 volumes of cold 95% ethanol. The precipitate was pelleted by centrifugation (10,000 rpm for 30 minutes at 4°C). The pellet was dried in a vacuum dessicator prior to resuspending it in 10 ml of part A buffer. Pre-digested protease K (see appendix) was added to a final concentration of 0.1 mg/ml and mixed by placing on an aliquot mixer for 60 minutes at room temperature. The solution was distributed to clean 30 ml corex tubes and was re-extracted 3 times with part B buffer. The nucleic acid was precipitated by adding 2 volumes of cold 95% ethanol, mixed and incubated at -20°C for at least 6 hours. The nucleic acid was pelleted by centrifugation (10,000 rpm for 20 minutes at 4°C). The vacuum dried pellet was dissolved in 5 ml of sterile distilled water. The RNA was preferentially precipitated by adding exactly 3 volumes of cold 4M sodium acetate, 10 mM EDTA, pH 6.0. The solution was mixed and incubated at 4°C for 10 minutes. After centrifugation (12,000 rpm for 30 minutes at 4°C), the pellet was resuspended in 5 ml of sterile distilled water. The RNA was reprecipitated by adding 1/10 volume of 3M sodium acetate, pH 5.0 and 2 volumes of cold 95% ethanol, The RNA was pelleted by centrifugation (10,000 rpm for 30 minutes at 4°C), vacuum dried and resuspeneded in 5 to 10 ml of sterile distilled water. A 5 µl aliquot of total RNA was diluted to determine the concentration in a spectrophotometer.

Poly(A)$^+$ RNA Isolation

The purification of polyadenylated RNA was achieved by using a mRNA purication kit (Pharmacia LKB Biotechnology). The pre-hydrated oligo (dT)-cellulose columns were loaded with 5 mg of total RNA dissolved in 1 ml of TE buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA). The poly(A)$^+$ RNA was sufficiently concentrated by two rounds of chromatography on each RNA sample. After purification, a 5 µl portion was removed and diluted in 1 ml of distilled water to determine the poly(A)$^+$ RNA concentration, using a spectrophotometer.

RNA Electrophoresis

The electrophoresis of RNA was achieved using the procedure described by Fourney *et al*,.(1988). All glassware, and the electrophoresis apparatus, were soaked in 0.1% (v/v) DEPC for at least 12 hours. Solutions and buffers intended for RNA usage were DEPC-treated prior to autoclaving. A 0.66 M formaldehyde, 1.8% agarose gel was prepared by adding the following to a baked Erlenmeyer flask: 3.6 g ultrapure agarose (BRL), 20 ml 10X MOPS/EDTA buffer (see appendix) and 174 ml of DEPC-treated water. The agarose was dissolved by heating prior to adding 10.2 ml of 37% deionized formaldehyde (in the fumehood; see appendix) when the solution cooled to about 50°C, the solution was gently mixed before the gel was poured into an RNase-free electrophoresis tray. After allowing the gel to set for 1 hour, running buffer (1X MOPS/EDTA; prepared from 10X MOPS/EDTA) was added to the gel box. Poly(A)$^+$ RNA (1 to 3 μg) or total RNA (10 to 30 μg) were added to separate RNase-free microcentrifuge tubes. The RNA volume was adjusted to 5 μl with sterile distilled water before adding 25 μl of electrophoresis sample buffer (see appendix) and 1 μl of ethidium bromide (1.0 mg/ml). The sample was denatured by heating at 65°C for 15 minutes prior to loading the gel. The RNA gels were run at 50 to 75 Volts (constant voltage) for 12 to 20 hours. After sufficient separation was achieved, a photograph was taken before northern blotting.

Radioactive Labeling of DNA

For all Southern blots, Northern blots and Filter-Colony hybridizations the ^{32}P-dCTP radioactive probes were made by the protocol described by Feinberg and Vogelstein (1983, 1984). DNA fragments used for probes were isolated by the electroelution method from agarose gels. Approximately 100 ng of DNA was used per lableling reaction and the volume was made up to 28 µl with TE buffer (10 mM Tris·HCl pH 7.8, 1 mM EDTA). The solution was placed in a boiling waterbath for 3 minutes and incubated on ice for 5 minutes. To this solution the following was added: 10 µl of OLB buffer (see appendix), 5 µl of $\alpha^{32}P$-dCTP (ICN Biomedicals, Inc., typically 50µCi), 5 µl of BSA (4 mg/ml) solution and 2 µl of Klenow fragment (1 unit/µl). After mixing thoroughly, the reaction was incubated at 37 to 42°C for at least 45 minutes. The oligo-labeling reaction was stopped by adding 100 µl to the stop buffer (20 mM NaCl, 20 mM Tris·HCl, pH 7.5, 0.25% (w/v) SDS). Non-incorporated radioactive label was removed on a Sephadex G-50 spin column equilibrated with TE buffer as described by Maniatis et al.,(1982). An aliquot (1 or 2 µl) was spotted on circular DE81 (Whatman) filter paper and washed twice with 0.3 M ammonium formate. The dried filter was placed in a scintillation vial containing 4 ml of aqueous counting scintillant (Amersham Corp.,Inc.) and the samples were counted in a Beckman 7500 liquid scintillation spectrophotometer.

Filter-Colony Hybridization

In filter-colony experiments the colonies were directly grown on selective medium. Sterile Bio Trans™ nylon membrane (pore size of 1.2 µm; ICN Biomedicals, Inc.) was overlayed onto the colonies according to the manufacturer's instructions. The transfered cells were lysed by placing the nylon membrane, colony side up, on 3 MM paper (Whatman) saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 4 minutes. Neutralization was achieved by placing the nylon membrane, colony side up, on 3 MM paper saturated with neutralizing solution (3 M sodium acetate, pH 5.5) for 5 minutes. The nylon membrane was air-dryed for 30 minutes prior to baking at 80°C for at least 1 hour.

The prehybridization and hybridization of Bio Trans™ nylon membranes essentially followed the manufacturer's procedure. Hybridization buffer (4 ml per 100 cm^2 of membrane; see appendix) was boiled for 5 minutes, cooled on ice for 5 minutes and added to a plastic bag containing the nylon membrane. Air bubbles were removed from the bag prior to sealing. The bag was immersed in a 42°C waterbath for at least 1 hour with gentle agitation. Radioactive label (one to two million cpm per ml) was denatured by placing in a boiling waterbath for 5 minutes. The denatured probe was immediately added to 4 ml of prehybridization buffer and mixed thoroughly. The mixture was added to the previous prehybridization solution. The bag was sealed and incubated in a 42°C waterbath for 5 to 24 hours with gentle shaking. After hybridization was completed, the membrane was removed from the bags and washed 3 times with 500 ml of 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% (w/v) SDS at room temperature. The nylon membrane was washed twice for 15 minutes at 50°C in 500 ml of 0.1X SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) 0.1% (w/v) SDS with gentle shaking. The membrane was removed from the washed buffer and covered with Saran Wrap^{TM} for autoradiography. X-ray film (XAR-5; Kodak) was exposed to the hybridized blots in an X-ray cassette (Picker) at -70°C. After sufficient exposure, the X-ray film was developed according to the manufacturer's instructions.

Southern Transfer

Southern transfer basically followed the procedure of Maniatis et al. (1982). After electrophoresis, the agarose gel was transfered to a glass baking dish and the unused portions of the gel were trimmed away. The DNA was denatured by soaking in several volumes of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 hour at room temperature with constant agitation. Neutralization of the gel was achieved by soaking in several volumes of neutralizing solution (3 M sodium acetate, pH 5.5) for 1 hour with gentle shaking at room temperature. After a brief rinse with distilled water, the gel was placed on a piece of Saran Wrap™. Bio Trans™ nylon membrane (0.2 µm; ICN Biomedicals, Inc.) was cut to size and overlayed onto the gel. A pasteur pipet was used to remove any bubbles between the agarose gel and the nylon membrane. Two pieces of 3 MM paper (Whatman) were cut to size and placed on top of the membrane. A stack of paper towels (5 to 7 cm high) was cut just smaller than the 3 MM paper and placed on top of the 3 MM paper. A weight (approximately 2 to 4 kg) was placed on the towels. The transfer of DNA was allowed to proceed for 12 to 24 hours. After blotting, the membrane was removed, air dried for 30 minutes and baked at 80°C for 1 hour. The prehybridization, hybridization, washes and autoradiography followed the procedure mentioned in the filter-colony hybridization section.

Northern Blotting

All glassware, solutions , and buffers intended for RNA usage were treated with DEPC as described in the RNA Electrophoresis section. Once electrophoresis was completed, the unused areas of the gel were removed by a razor blade. The transfer of RNA to Bio Trans™ nylon membrane followed the procedure described by Fourney et al. (1988). The gel was prepared for transfer by soaking it twice for 20 minute periods in 10X SSC (1.5 NaCl, 0.3 M sodium citrate, pH 7.0) at room temperature with gentle agitation. The nylon membrane (cut to size) was pre-wet with distilled water for 5 minutes prior to a 5 minute soak in 10X SSC. The transfer efficiency and time was decreased by using a polyurethane sponge to enhance capillary action. The sized sponge was pre-soaked in 10X SSC before placing in a glass baking dish. Six pieces of 3 MM paper (Whatman) were cut to the same size of the fomaldehyde-agarose gel. Three pieces of the 3 MM paper were placed on top of the sponges followed by the northern gel. The sized pre-soaked nylon membrane was overlayed onto the gel followed by the other 3 pieces of 3 MM filter paper. A stack of paper towels (10 to 15 cm high) was cut slightly smaller than the 3 MM paper and placed on top of the filter paper. A book (~ 2 kg) was placed onto the stack of towels for weight and blotting was allowed to proceed for at least 24 hours. When RNA transfer was complete the nylon membrane was removed. The nylon was air dried for 30 minutes prior to baking at 80°C for at least 1 hour. The prehybridization, hybridization, washes and autoradiography followed the procedure described in the filter-colony hybridization section.

In Vivo Protein Labeling

Neurospora cultures were grown in liquid Vogel's medium (see appendix) inoculated at a concentration of 1 to 5 million conidia per ml. Labeling of cells was achieved by a modification of the procedure described by Hallermeyer *et al.* (1977). Labeling with L-^{3}H-leucine (Amersham Corp, 52 Ci/mmol) or L-^{35}S-methionine (Amersham Corp, 1120 Ci/mmol) was accomplished by growing the cultures to log phase in 100 ml of liquid Vogel's medium, then adding 1 mCi of either radiosotope. The cultures were grown for an additional 3 hours before harvesting the cells by filtration. The mycelial pad was washed two times with ice-water and placed in a mortar. To every gram of mycelium the following was added: 1.5 g of acid-washed sand (see appendix) and 1 ml of 15% grinding buffer (15% (w/v) sucrose, 50 mM Tris.HCl, pH 7.8, 0.25 mM EDTA). The mixture was ground with a pestle. After sufficient grinding, the suspension was transferred to SS-34 (Dupont) tubes. The sand was pelleted by centrifugation (4,000 rpm for 5 minutes at 4°C) The supernatant was carefully decanted to a clean SS-34 tube and any remaining sand and cell debris were pelleted by a second centrifugation as above. The supernatant was poured to a fresh SS-34 tube. The mitochondria were pelleted by centrifugation (12,000 rpm for 20 minutes at 4°C) The crude mitochondrial pellet was resuspended in 250 µl of 0.1 M Tris·HCl , pH 7.5, 0.01 M EDTA. A 5 µl aliquot was used to count the specific activity of the sample in a Beckman LS7500 liquid scintillation counter. The radioactive mitochondrial solution was stored at -20°C.

In Vitro Translation

In vitro translation was achieved using a rabbit reticulocyte system (Bethesda Research Laboratories). The radioactive label used in the system was L-^{35}S-methionine (Amersham, 1120 Ci/mmol). The actual *in vitro* translation reaction followed the manufacturer's instructions. In addition to *Neurospora* poly(A)$^+$ mRNA, rabbit globin mRNA (BRL, Inc.) was used to provide a control. To determine the extent of incorporation two 5 µl samples of each reaction were removed and added to two plastic scintillation vials containing 1 ml of distilled water. To each vial, 0.5 ml of decolorizing solution (1 M NaOH, 0.5 M H_2O_2, 1 mg/ ml L-methionine) was added (Pelham and Jackson, 1976). The solutions were mixed thoroughly by shaking and incubated for 15 minutes at 37°C. The proteins were precipitated by adding 1 ml of 25% (w/v) trichloroacetic acid (TCA). The precipitate was collected on a GFC filter (Whatman). The filters were washed three times with 5% (w/v) TCA for two minutes each and two times with 95% ethanol in 125 ml Erlenmeyer flasks. The filters were allowed to air dry before adding them to scintillation vials containing 4 ml of aqueous counting scintillant. The counts per minute were recorded for later reference.

Immunoprecipitation of Holocytochrome *c* and apocytochrome *c*

For holocytochrome *c* immunoprecipitation, 100 µl of antiserum was added to 100 µg of mitochondrial protein containing 100,000 to 300,000 cpm of ^{35}S or 1 to 2 million cpm of ^{3}H. In one experiment, mitochondria from wild-type (NCN 10; labeled with L-^{35}S-methionine) and *cyt-12-1* (NCN 82; labeled with L-^{3}H-leucine) strains were mixed together. The actual immunoprecipitation and the washing of the immunoprecipitates were as described by Hennig and Neupert (1983) utilizing protein A-sepharose, except that the final pellets were resuspended in cracking buffer (5% SDS, 5% beta-mercaptoethanol, 100 mM Tris-HCl, pH 7.5, 10% glycerol, 0.01% bromphenol blue). The protein concentration of the mitochondrial suspensions used in the immunoprecipitation procedure were determined by the dye binding assay (Bio-Rad; Bradford, 1976).

The immunoprecipitation of apocytochrome *c* was achieved directly from *in vitro* translation reactions. The mixture, corresponding to 1 to 2 million cpm of incorporated ^{35}S-methionine, was made to 2 or 3% with SDS and boiled for 5 minutes. The above step was performed to minimize aggregation of cytochrome *c* into dimers and tetramers. The SDS concentration was then diluted to 0.1% by the addition of dilution buffer (10 mM Tris.HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2% Triton X-100). Antiserum (100 µl) raised against apocytochrome *c* was mixed with 8 mg of protein A-Sepharose complex (pre-hydrated in dilution buffer) on an aliquot mixer for at least 1 hour at 4°C. The antibody/protein A-Sepharose complex was washed with 1 ml of dilution buffer before adding to the diluted lysate mixture. After incubating for 3 hours on an aliquot mixer at 4°C, the immunoprecipitates were pelleted by centrifugation (2 minutes in an Eppendorf microcentrifuge). The pellet was washed 3 times with dilution buffer and 2 times with dilution buffer without Triton X-100. The pellet was resuspended in cracking buffer and boiled for 5 minutes.

After centrifugation, the supernatant was electrophoresed as described in the protein electrophoresis section (below). The antiserum raised against holocytochrome *c* and apocytochrome *c* was a kind gift from Drs. Rosemary Stuart, Christoph Hergersberg, and Walter Neupert, Institut for Physiologische Chemie, University of Munich.

Protein Electrophoresis

Electrophoresis of the immunoprecipitates to quanititate holocytochrome *c* in mitochondria or apocytochrome *c* from *in vitro* translation was essentially as described (Weiss et al.1971; Bertrand and Werner, 1977; Hames and Rickwood, 1981) except that vertical electrophoresis (3 mm thick dis-continuous SDS-PAGE gels). The stacking gel and the separation gel were 3.75% and 10% acrylamide, respectively.

After electrophoresis, the gel was fixed and stained in several volumes of methanol/water/acetic acid (4:5:1) containing 0.25% Coomassie Blue R-250, for 30 minutes with gentle shaking. The gel was then destained by soaking in several volumes of destain (methanol/water/acetic acid (5:4:1)) for 4 hours with gentle agitation, and one subsequent additional change of destain. After destaining, the gel was soaked in Amplify (Amersham Corp) for 40 minutes. The protein gel was dried in a Bio-Rad Slab Dryer at 60°C for several hours. Autoradiography of the dried gel was performed at -70°C using XAR-5 X-ray film (Kodak) for 10 hours to several days, depending upon the band intensities. The film was developed according to the manufacturer's instructions.

Alternatively, acrylamide gels for the quantitation of some holocytochrome *c* and all apocytochrome *c* experiments gels were sliced into 1.1 mm slices by a gel slicing apparatus (Tyler Instruments, Inc.) and placed into plastic scintillation vials containing 750 µl of 1% (w/v) SDS, 0.1M Tris·HCl, pH 8.0. The proteins were eluted by heating for 9 to 12 hours at 65°C. After the incubation, 4 ml of aqueous scintillation fluid (Amersham Corp.) was added to each vial before recording the number of counts in a scintillation counter.

Enzymes

All enzymes used in this study were used according to the supplier's instructions.

III.RESULTS

Isolation of Cosmid Clones by Sib-Selection

The *cyc-1-1* mutant was isolated by Pittenger and West (1979) and shown to be deficient in cytochrome *c*. The initial goal of this work was to isolate and characterize the gene affected in this mutant. To achieve this, cosmids containing the *cyc-1*$^+$ gene were isolated by sib selection from the wild-type *N.crassa* (strain *74A*) genomic cosmid library described by Drygas *et al.*(1989). This library contains *N.crassa* genomic DNA in the vector pSV50 (Fig.4) and is maintained in 3168 separate colonies of *E.coli* DH1 stored at -70°C in microtiter dishes. This library was examined for the ability to rescue the slow-growth character of *cyc-1-1* according to the sib selection procedure described by Akins and Lambowitz (1985). Sphaeroplasts of the *cyc-1-1* mutant were transformed with purified cosmid DNA, from *E.coli* cultures containing pooled clones. Sibs capable of complementing the *cyc-1-1* mutant yielded fast-growing, benomyl resistant, transformants against a background of slow-growing non-rescued colonies transformed only to benomyl resistance. Non-complementing sibs showed only slow-growing benomyl resistant colonies. After 4 rounds of transformation using sib selection, a single cosmid, pSV50-5324, was identified as containing the *cyc-1*$^+$ gene that rescued the *cyc-1-1* mutant strain (Fig.5).

Cosmid Sub-cloning

Sub-cloning the *cyc-1*$^+$ gene from the cosmid pSV50-5324 was necessary for the characterization of the gene by DNA sequence analysis, since the cosmid contained *ca*. 36 kb insert of *N.crassa* genomic DNA. To identify which restriction enzymes cut the *cyc-1*$^+$ gene, cosmid (pSV50-5324) DNA was initially digested with several restriction enzymes and a small aliquot of each digest was electrophoresed on a 0.8% agarose gel to confirm complete digestion. The remaining digested DNAs (approximately 2µg) were combined with an uncut sample of pSV50 (0.5µg), to provide an intact benomyl resistance gene, and used to cotransform *cyc-1-1* sphaeroplasts. The transformation results indicated that the enzymes EcoRI, HindIII, PstI, SphI, SstI, and XmaI do not cut the *cyc-1*$^+$ gene because fast-growing, benomyl resistant, transformants were observed (Table 5). However, the restriction enzymes BglII, KpnI, SalI, and XhoI did cut the *cyc-1*$^+$ gene, since transformations with these did not yield fast-growing transformants (Table 5). Transformation results revealed that the enzyme BamHI did not fall clearly into either group, because transformations with BamHI digested pSV50-5324 gave fewer transformants than transformations with pSV50-5324 digested with enzymes that do not cut the wild-type gene.

To determine which digests would be most useful for subcloning the *cyc-1*$^+$ gene, the electrophoretic patterns of pSV50-5324 separately digested with the restriction endonucleases PstI, HindIII, EcoRI, and BamHI (Fig.6, lanes N, P, R, and T respectively), were analysed. Following electrophoresis it was judged that the 8 fragments, from a PstI digest of pSV50-5324, would be most easily isolated by electroelution. Each fragment (approximately 1µg), along with the vector pSV50 (0.5µg), was used to cotransform *cyc-1-1* sphaeroplasts. The transformation results indicated that the largest fragment (PstI-1, approximately 13 kb in length) contained the *cyc-1*$^+$ gene. Subsequently, purified PstI-1 fragment was digested with HindIII, EcoRI, and SphI and analysed by agarose gel electrophoresis (not shown). The SphI digest of PstI-1 fragment yielded two easily separable fragments (10 kb and 3 kb in length). These two fragments were purified from a 0.8% agarose gel by electroelution and used with pSV50 to cotransform *cyc-1-1* sphaeroplasts as described above. Fast-growing, benomyl resistant, transformants were observed when the 10 kb PstI-SphI fragment was used to cotransform *cyc-1-1* sphaeroplasts.

During the course of the above work, others in the laboratory were performing similiar experiments on the *cyt-12-1* mutant of *N.crassa* and had isolated a cosmid clone (pSV50-4333) from the same genomic library described above, capable of rescuing the slow-growth character of the mutant. A 3.9 kb HindIII-PstI fragment, containing the *cyt-12*$^+$ gene, was sub-cloned from pSV50-4333 into the pUC19 vector. The plasmid clone, designated pSP8 (see Figure 7 for partial restriction map), when used in a cotransformation with pSV50 DNA, was able to rescue *cyt-12-1* sphaeroplasts to fast-growth and benomyl resistance.

The *cyt-12-1* mutant is characterized by a deficiency of cytochrome *c* and a partial deficiency of cytochrome *aa*$_3$ (Bertrand *et al.*,1977). When initially isolated, the *cyt-12-1* and *cyc-1-1* mutants were classified into the basic groups "cyt" and "cyc" based on their phenotypic differences: deficiency of more than one cytochrome, and deficiency of only cytochrome *c*, respectively. In genetic crosses, the two mutants were shown to be linked to similiar markers on linkage group II (Bertrand *et al.*, 1977; H. Bertrand, personal communication). At about the time that *cyt-12*$^+$ was cloned, it was also shown in our laboratory that the mutation in the *cyt-2-1* strain of *N.crassa*, which is also deficient in both cytochromes *aa*$_3$ and *c*, affected the enzyme cytochrome *c* heme lyase (Drygas *et al.*, 1988). These results proved that gross defects in cytochrome *c* metabolism could lead to, by an unknown mechanism, gross deficiencies in cytochrome *aa*$_3$. Thus, it was suspected that the defect in *cyt-12-1* might also affect only cytochrome *c* metabolism and that *cyt-12-1* and *cyc-1-1* might be alleles of the same gene, given their proximity on the genetic map. It was then discovered that complementation studies between *cyt-12-1* and *cyc-1-1* had never been performed (H. Bertrand, personal communication) and that *cyc-1-1* was also deficient in cytochrome aa_3 if grown at high temperature (H. Bertrand, personal communication; D.A. Bottorff, F.E. Nargang, unpublished results). Therefore, an examination of the relationship between *cyc-1-1* and *cyt-12-1* was undertaken, which consisted of a complementation test; reciprocal transformation experiments involving the two mutants and the clones pSP8, pSV50-4333, and pSV50-5324; and detailed restriction and southern analysis of the two cosmid clones.

Complementation Analysis Using Heterokaryons

Heterokaryons were forced by superimposing conidia from strains of *cyt-12-1* and *cyc-1-1*, with different nutritional requirements on minimal media. Cytochrome spectral analysis of mitochondria isolated from several *cyt-12-1*/*cyc-1-1* heterokaryons showed that the two mutant strains do not complement each other since the heterokaryons are deficient in cytochrome *c* (Fig.8). These results strongly suggested that *cyc-1-1* and *cyt-12-1* are allelic mutations of the same gene and it was apparent that further characterization of the cosmids, pSV50-4333 and pSV50-5324, was necessary to determine if both contain the same gene capable of rescuing *cyc-1-1* and *cyt-12-1*.

Reciprocal Transformations of *Cyc-1-1* and *Cyt-12-1*

Purified DNA, from either pSV50-4333, pSV50-5324, or pSP8 plus pSV50 was used to transform sphaeroplasts of both *cyc-1-1* and *cyt-12-1* in separate experiments. Many fast-growing, benomyl resistant, transformants were observed in all 6 transformations (transformation frequencies were not determined). Several of the fast-growing colonies were isolated and grown in separate conidia flasks containing benomyl. Conidia from these flasks were used to inoculate liquid Vogel's medium containing benomyl and spectral analysis of mitochondria isolated from these cultures provided evidence that both cosmids and the recombinant plasmid (pSP8) rescue both cytochrome *c* deficient strains *cyc-1-1* and *cyt-12-1* (Fig.9) / Higher levels of cytochrome *c* and beta-peaks were observed in all transformants as compared to the untransformed parental strains. The higher beta-peaks might be the result of not determining spectra immediately after harvesting the cultures; however, this was not studied further.

Restriction and Southern Analysis of the Cosmids

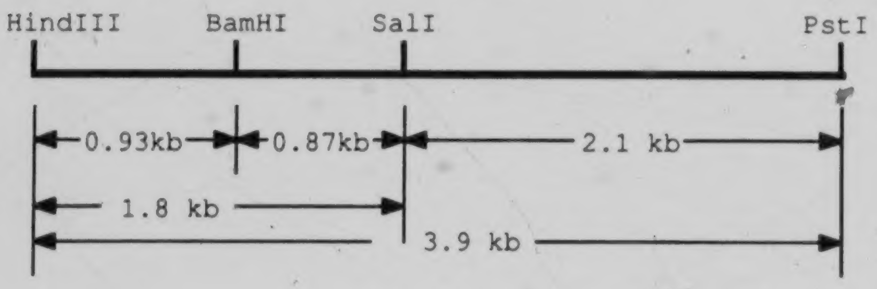
To examine the similiarity between the two cosmids, pSV50-4333 and pSV50-5324, and between pSV50-5324 and plasmid pSP8, the isolated DNAs were digested with several restriction enzymes and analysed by gel electrophoresis (Fig.6). The restriction patterns of several digests of both cosmids indicated that many fragments appeared to be in common since they have similar migration rates (Fig.6, compare lanes B and C, F and G, J and K, L and M, N and O, P and Q, R and S, or T and U). It was noted in an earlier section that the 3.9 kb HindIII-PstI fragment, that contains the *cyt-12*$^+$ gene, was subcloned from pSV50-4333 into pUC19 to yield the clone pSP8. Plasmid DNA (pSP8) was digested with the enzymes HindIII and PstI (Fig.6, lane H) and compared to pSV50-5324 cut with the same enzymes (Fig.6, lane F). The number of bands and their proximity to each other in these digests made it difficult to determine with certainty if the 3.9 kb band present in pSP8 was also present in pSV50-5324. Therefore, the cosmid and plasmid DNAs (Fig.6) were further analysed by southern analysis, utilizing ^{32}P-labelled 3.9 kb HindIII-PstI fragment, purified from pSP8, as the probe (Fig.10). The probe hybridized to a 3.9 kb fragment from HindIII/PstI digests of both pSV50-4333 and pSP8 as expected (Fig.10, lanes G and H, respectively). Surprisingly, the probe only detected a 2.3 kb HindIII-PstI fragment of pSV50-5324 (Fig.10, lane F).

Earlier transformations of *cyc-1-1* and *cyt-12-1* sphaeroplasts, with either cosmid digested with the enzyme SalI, indicated that SalI cuts both wild-type genes, since the transformants are rescued only to benomyl resistance (see Cosmid Sub-cloning for *cyc-1*$^+$; *cyt-12*$^+$ data not shown). Restriction analysis of several digests of pSP8, using various combinations of the enzymes HindIII, PstI and SalI, showed that SalI cuts the 3.9 kb HindIII-PstI genomic fragment into a 1.8 kb HindIII-SalI and a 2.1 kb SalI-PstI fragment (not shown). Cosmid DNA (pSV50-5324) was digested with HindIII, PstI, and SalI and the restriction pattern was compared to the same digest of pSP8 (Fig.6, compare lanes B and D, respectively) Unfortunately, due to the number of bands present in the pSV50-5324 digest, it could not be ascertained with certainty whether or not pSV50-5324 contained the 1.8 kb HindIII-SalI and/or the 2.1 kb SalI-PstI fragments. When the above digests were analysed by southern analysis using the 3.9 kb HindIII-PstI fragment of pSP8 as a probe, it was found that the probe hybridized to three fragments, 2.3 kb, 1.8 kb, and 490 bps in length from the HindIII/PstI/SalI digest of pSV50-5324 (Fig.10, lane B). The lower intensity of the 2.3 kb band and the fact that it corresponded to the sum of the other two fragments suggested that it was the result of incomplete digestion by SalI. Given the above assumption, the fact that the same probe detected a 2.3 kb fragment from HindIII and HindIII/PstI digests of pSV50-5324 (Fig.10, lanes P and F, respectively) and a 490 bp fragment from pSV50-5324 digested with HindIII, PstI, and SalI, suggested that a SalI restriction site was located approximately 500 nucleotides away from one of the HindIII sites (2.3 kb HindIII-HindIII fragment minus the previously determined 1.8 kb HindIII-SalI fragment length).

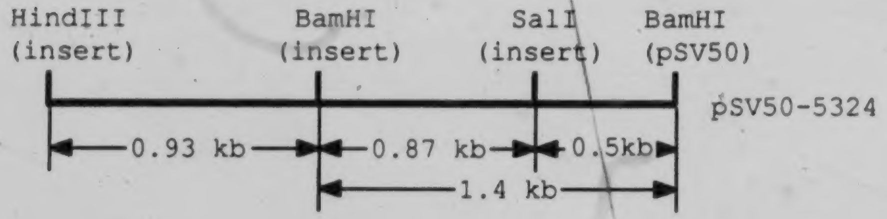
Although it had been shown that pSV50-5324, pSV50-4333, and pSP8 all carried the *cyt-12*$^+$ (or *cyc-1*$^+$) gene (see Reciprocal transformations), the analysis described above showed that the region of genomic DNA carrying the *cyt-12*$^+$

gene and/or its flanking region in cosmid pSV50-5324 was not identical to that in cosmid pSV50-4333 (or its subclone, pSP8). A reasonable explanation for this observation was that one cosmid contains the *cyt-12*$^+$ gene on a region of genomic DNA close to one junction of insert and vector sequences. Therefore, further analysis was performed by taking the location of restrictions sites in the pSV50 vector into consideration. The BglII site in the polylinker region of pSV50 was the site in which the Sau3AI partially digested genomic DNA fragments were inserted to construct the library (Vollmer and Yanofsky, 1986). This site is flanked by two BamHI, two EcoRI, and one HindIII site (see Figure 4). One possible explanation for the results described above was that the HindIII site shown to be 0.5 kb away from a SalI site in pSV50-5324, was in fact the pSV50 HindIII site.near the insertion/vector boundary. Furthermore, given the proximity of the BamHI site in the pSV50 polylinker to the nearby HindIII site, a subsequent prediction would be the existance of a *ca*. 0.5 kb SalI-BamHI fragment in pSV50-5324.

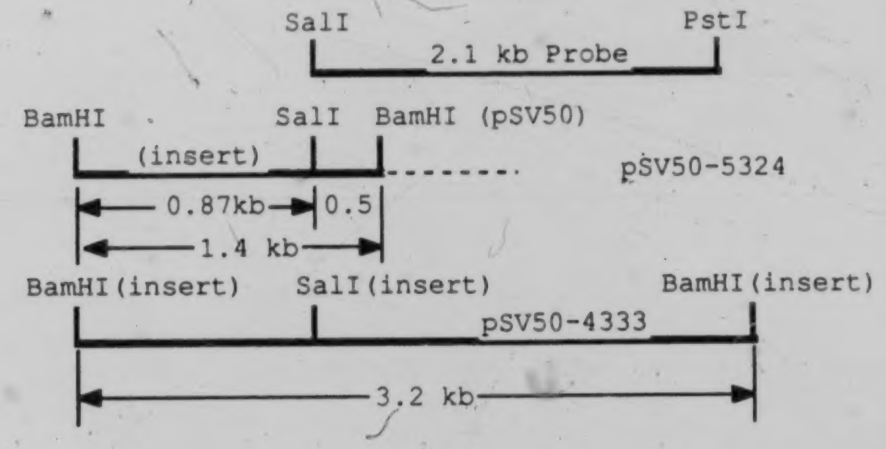
Restriction analysis of pSP8 digested with BamHI indicated that BamHI cuts the genomic insert once, yielding a 930 bp HindIII-BamHI fragment and a 3 kb BamHI-PstI fragment (not shown). The size of the BamHI-SalI fragment was calculated to be 0.87 kb (1.8 kb HindIII-SalI minus the 0.93 kb HindIII-BamHI distance) and subsequently was confirmed by restriction analysis (not shown). So that the arrangements of sites in the pSP8 insert (and the corresponding region of pSV50-4333) would be:


HindIII
BamHI
SalI
PstI
0.93kb
0.87kb
2.1 kb
1.8 kb
3.9 kb

When both cosmids were examined, it was found that the 3.9 kb HindIII-PstI probe hybridized to a 3.2 kb fragment and to a *ca.* 25 kb BamHI fragment from pSV50-4333 (Fig.10, lane U), while 1.4 kb and *ca.* 25 kb fragments were detected from BamHI digests of pSV50-5324 (Fig.10, lane T). It was suspected that the 1.4 kb BamHI fragment of pSV50-5324 corresponded to the 0.87 kb BamHI-SalI fragment plus the proposed 0.5 kb SalI-BamHI fragment at the insert/vector boundary (see above). In order to prove this suggestion, additional analysis was done using the 1.8 kb HindIII-SalI fragment and the 2.1 kb SalI-PstI fragment from the insert

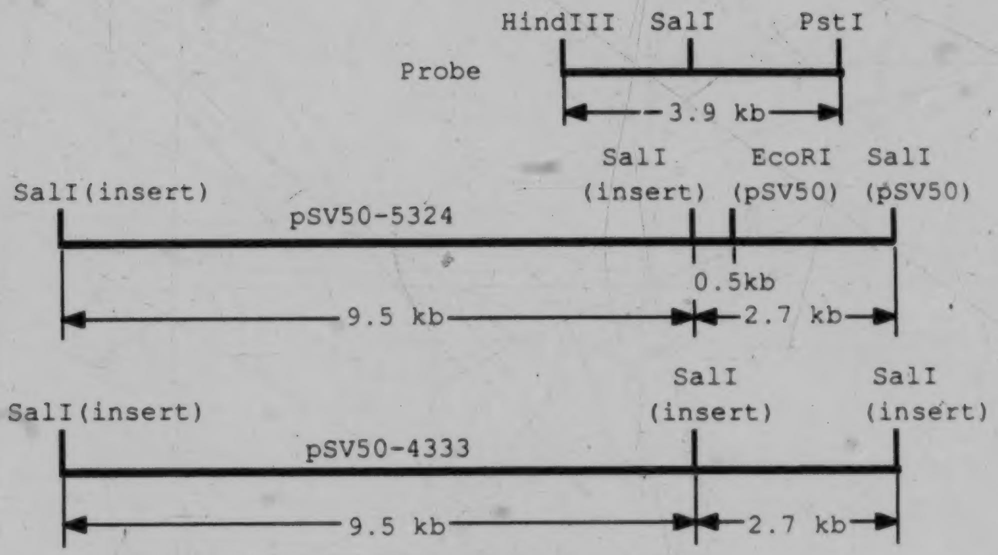

HindIII (insert)
BamHI (insert)
SalI (insert)
BamHI (pSV50)
pSV50-5324
0.93 kb
0.87 kb
0.5kb
1.4 kb

region of pSP8 as probes. Both cosmids were digested with various enzymes, analysed by gel electrophoresis (Fig.11), and further analysed by southern analysis. The southern blot was probed with ^{32}P-labelled 1.8 kb HindIII-SalI fragment (Fig.12A), stripped, and reprobed with ^{32}P-labelled 2.1 kb SalI-PstI fragment (Fig.12B). The 1.8 kb HindIII-SalI probe hybridized to the two BamHI fragments in each of pSV50-4333 and pSV50-5324 (Fig.12A, lanes J and H respectively) previously described using the entire 3.9 kb HindIII-PstI fragment as a probe (Figure 10, lanes U and T, respectively). Therefore, it was predicted that the 2.1 kb SalI-PstI probe should hybridize to the 1.4 kb BamHI fragment of pSV50-5324, since the probe is homologous to about 0.5 kb of the 1.4 kb fragment, and to the 3.2 kb


SalI
PstI
2.1 kb Probe
BamHI
SalI
BamHI (pSV50)
(insert)
pSV50-5324
0.87kb
0.5
1.4 kb
BamHI(insert)
SalI(insert)
BamHI(insert)
pSV50-4333
3.2 kb

BamHI fragment of pSV50-4333 (see above). The observed results verified these predictions, since the 2.1 kb SalI-PstI probe detected the 1.4 kb and 3.2 kb BamHI fragments of pSV50-5324 (Fig.12B, lane H) and pSV50-4333 (Fig.12B, lane J),respectively. Thus, these results support the notion that in addition to the HindIII site (described earlier), a BamHI site also exists about 0.5 kb from the SalI site located in the *cyt-12*$^+$ gene of pSV50-5324, but not in pSV50-4333.

The above arrangment of restriction sites in pSV50-5324 would also lead to the prediction that a pSV50 EcoRI site should be present in the vicinty of the pSV50 BamHI and HindIII sites (see Fig.4). Restriction analysis of pSP8, revealed that EcoRI does not cut the 3.9 kb HindIII-PstI genomic fragment (not shown). The 3.9 kb HindIII-PstI probe hybridized to two fragments, 0.5 kb and 9.5 kb, from an EcoRI/SalI digest of pSV50-5324 (Fig.10, lane J), while 2.7 kb and 9.5 kb fragments were detected from the same digest of pSV50-4333 (Fig.10, lane K). The HindIII-PstI probe detected both fragments, 2.7 kb and 9.5 kb, from SalI digests of pSV50-5324 and pSV50-4333 (Fig.10, lanes L and M, respectively), but the band intensity of the 2.7 kb fragment detected from pSV50-5324 was faint. This was probably due to the fact that the 3.9 kb HindIII-PstI probe and the 2.7 kb fragment of pSV50-5324 have only a small region in common (*ca*. 0.5 kb), in comparison to the 2.7 kb fragment detected from pSV50-4333 (2.1 kb SalI-PstI similarity) (see below).


HindIII
SalI
PstI
Probe
3.9 kb
SalI (insert)
pSV50-5324
SalI (insert)
EcoRI (pSV50)
SalI (pSV50)
0.5kb
9.5 kb
2.7 kb
SalI (insert)
pSV50-4333
SalI (insert)
SalI (insert)
9.5 kb
2.7 kb

These results confirm the prediction that an EcoRI restriction site exists about 0.5 kb away from the SalI site in pSV50-5324. The presence of all 3 restriction sites, BamHI, HindIII, and EcoRI, in the same region provide good evidence that vector pSV50 sequences lie adjacent to the *cyt-12*$^+$ gene in pSV50-5324, resulting in the restriction pattern differences observed between the two cosmid clones.

The orientation of pSV50 relative to the 3.9 kb HindIII-PstI fragment, containing the *cyt-12*$^+$ gene, in the cosmid clone pSV50-5324 was also determined. Analysis of the restriction map of pSV50 (Fig.4), showed that pSV50 contained a SalI restriction site approximately 2 kb away from the polylinker region in one direction and a SalI restriction site 3 kb in the other orientation. The 1.8 kb HindIII-SalI probe detected a 9.5 kb SalI-SalI fragment from both cosmids (Fig.12A, lanes A and B), while the 2.1 kb SalI-PstI fragment as probe detected a 2.7 kb fragment from separate SalI digests of pSV50-5324 and pSV50-4333 (Fig.12B, lanes A and B, respectively). The fact that both probes, 2.1 kb SalI-PstI and 3.9 kb HindIII-PstI (mentioned earlier), detected a 2.7 kb SalI fragment from pSV50-5324 suggested that the SalI site is approximately 2 kb from the polylinker region (2.7 kb minus the SalI-polylinker distance of 500 bp) and that the COS region of pSV50 is proximal to the *cyt-12*$^+$ gene.

All of the southern analysis data was subsequently used to construct partial restriction maps of the cosmids pSV50-4333 and pSV50-5324 (Fig.13). The order of the two restriction sites BamHI and EcoRI located *ca*. 25 kb away from the *cyt-12*$^+$ gene could not be determined because of their similar migration rates. Therefore, this EcoRI site was not shown in the separate partial restriction maps of the two cosmids.

DNA Sequence Analysis of the *Cyt-12*$^+$ Gene

For the reasons described in the above 3 sections, it was concluded that *cyc-1-1* and *cyt-12-1* were allelic mutations. Note that a suggestion for renaming these alleles of the same gene is given in the Discussion. The DNA sequence of the *cyt-12*$^+$ gene rather than *cyc-1*$^+$ was determined, since a small fragment containing this gene had already been subcloned. Various *cyt-12*$^+$ fragments, isolated from the plasmid pSP8, were subcloned into the M13 vectors mp18 and mp19 which facilitated the isolation of single-stranded template for DNA sequence analysis. The *cyt-12*$^+$ 1.8kb HindIII-SalI and 2.1kb SalI-PstI fragments (see Fig.6,lane D) were subcloned into M13 phage vectors and designated SPA and SPB, respectively. DNA sequence analysis of these phage clones showed that the predicted amino-acid sequence corresponded to the previously determined protein sequence of *N.crassa* cytochrome *c* (Heller and Smith, 1966; Lederer and Simon, 1974).

During the initial stages of the DNA sequence analysis of the *cyt-12*$^+$ gene, Stuart *et al.*(1987) published their work on the cloning and sequencing of cytochrome *c* cDNAs from wild-type (*74A*) and the *cyc-1-1* mutant. Their analysis revealed that the mutation in *cyc-1-1* affects an intron/exon splice site junction of the 2nd intron and results in the *cyc-1-1* cytochrome *c* protein being 19 amino acids longer than wild-type, with the terminal 27 amino acids being unrelated to the wild-type sequence. Even though the cytochrome *c* cDNA and the wild-type genomic sequence had been determined in the mutant splice site region, it was decided that the sequence of the entire genomic cytochrome *c* gene would be of interest, since it would reveal the 5' upstream sequences and intronic sequences.

DNA sequence analysis of the two clones SPA and SPB revealed other enzymes that could be utilized for subcloning the *cyt-12*$^+$ gene into M13 vectors. The DNA sequencing strategy and the partial restriction map of the *cyt-12*$^+$ gene is shown in Figure 14. The Dale method of single-stranded M13 subcloning was used to obtain some sequence of the *cyt-12*$^+$ gene as described in Materials and Methods. The sequence of the coding strand and the amino acid sequence of the protein is given in Figure 15. The numbering system is relative to the translation start site which was assigned the position +1. Sequence analysis of the cytochrome *c* gene revealed that the restriction endonucleases (BglII, KpnI, SalI, and XhoI) that gave little, if any, transformants during the course of subcloning the *cyt-12*$^+$ gene from pSV50-5324, have recognition sites within the gene, while the enzymes (EcoRI, HindIII, SphI, SstI, and XmaI) that yielded many transformants were found not to occur in the sequence of the *N.crassa* cytochrome *c* gene as expected. DNA sequence analysis of the wild-type (*74A*) cytochrome *c* cDNA revealed that the transcription initiation site was probably located at position -104 , since the cDNA sequence ended at that position (Stuart *et al.*, 1987). However, no further experiments such as 5' primer extension analysis of mRNAs were subsequently undertaken by Stuart *et al.*(1987) to confirm this observation. The residue frequencies of the *cyt-12*$^+$ gene (Table 6) showed that the *cyt-12*$^+$ coding region was slightly (G + C) rich (58.3%).

DNA sequence analysis of the *cyt-12*$^+$ gene and comparison to the known amino acid and cDNA sequences revealed that the wild-type *N.crassa* cytochrome *c* gene consisted of 3 exons and 2 introns. It was determined that exons 1, 2, and 3 encoded 5, 96, and 7 amino acids, respectively, of the cytochrome *c* gene (total being 108 amino acids in length). Intron 1 was 543 nucleotides in length, while intron 2 was 71 base pairs long. Ballance (1986) analysed the DNA sequence from a number of genes in filamentous fungi and yeast and observed that certain consensus sequences at RNA splice sites and translation initiation sites could be found. The 5' exon-intron splice sites of the *cyt-12*$^+$ gene, G|GTATGTA (starting at position 17) of intron 1 and C|GTACGTC (position 850) of intron 2, closely resembled the same consensus sequences determined from filamentous fungi (g|GTAYGTPy) and yeast (N|GTATGT) genes. The 3' intron-exon boundary sequences, CCAGG (position 561) of intron 1 and ACAGG (position 921) of intron 2 of *cyt-12*$^+$, were also found to be similiar to the 3' splice site consensus sequences of filamentous fungi and yeast genes (ACAG|g and YYNYAG|N, respectively). The RNA splicing signals for lariat formation, TTCTAAG (starting at position 540 of intron 1) and AGCTAAC (position 899; intron 2) of the wild-type cytochrome *c* gene, matched the consensus sequence for the site of lariat formation in yeast and filamentous fungi genes. The sequences surrounding the AUG start codon (CAGTCAAAAUGGG) of the *cyt-*

12$^+$ gene resembled the same region observed in several *Neurospora* genes (CNATCACAAUGNC).

The sequence, AAGAAA, that occurs 17 nucleotides upstream from the site of polyadenylation of the *N.crassa* cytochrome *c* gene may be the polyadenylation signal (Stuart *et al.*, 1987). Similar sequences were also found in other *N.crassa* genes such as *con-10* (AAAAAGA, 19 nucleotides from the end of the transcript; Roberts *et al.*, 1988) and the nuclear encoded proteolipid subunit of mitochondrial ATPase (AAAAAGAAA, 20 nucleotides from the end of the transcript; Viebrock *et al.*, 1982). However, no experimental evidence has shown that these sequences are indeed the signals for polyadenylation.

Codon usage analysis of the *cyt-12*$^+$ gene (Table 7) revealed that *N.crassa* cytochrome *c* protein was slightly basic. There were 14 lysines and 3 arginines versus 6 glutamates and 7 aspartates, an excess of 4 basic residues in a protein of 108 residues. Pyrimidines are used 3 times more frequently than purines in the 3rd position of codons. The strong preference (83.5%) for a G or C residue used in the wobble position of *cyt-12*$^+$ gene codons is similiar to the codon usage of other *N.crassa* nuclear genes (Sachs *et al.*, 1989). The wild-type cytochrome *c* gene has a bias against A in the 3rd position (0.9%) of codons that is often observed in highly expressed *N.crassa* genes such as beta-tubulin (0.9%; Orbach *et al.*, 1986), *am* (0.2%; Kinnaird and Fincham, 1983) and histones H3 and H4 (0.7%

and 2%, respectively; Woudt *et al.*, 1983). This bias suggested that the *cyt-12*$^{+}$ gene could be considered to be expressed at a high level.

The upstream region of the *cyt-12*$^{+}$ gene was examined for sequences that could be involved in gene regulation. Two sequences of the *cyt-12*$^{+}$ gene (Table 8), starting at positions -146 and -160, were found that might be related to upstream activation sequences (UAS) observed in the yeast genes CYC1 (Guarente *et al.*, 1984), CYC3 (Dumont *et al.*, 1987), and CTT1 (Spevak *et al.*, 1986). These yeast genes are known to respond to different concentrations of hemin and catabolites. Three related upstream sequences of the *N.crassa cyt-2*$^{+}$ gene (Drygas *et al.*, 1989), are also shown in Table 8. However, the similiarity of these sequences, even between the different yeast genes, is not exceptional. Furthermore, no sequences have been shown to function as UAS in *Neurospora*. Thus, the potential significance of the regions should be viewed with considerable skepticism until functional analysis has been done.

The *cyt-12*$^{+}$ upstream region was also compared to the upstream sequences of the *N.crassa* genes *cyt-21* (Kuiper *et al.*, 1988), *cyt-18* (Akins and Lambowitz, 1987); cyclophilin (cycph) (Tropschug *et al.*, 1988), *mrp-3* (Kreader *et al.*, 1989), and *cya-4* (Sachs *et al.*, 1989) whose products are also found within mitochondria . Several short regions of homology (>80%, min. of 10 matches, max. loopout of 2 nucleotides) were found between the upstream region of *cyt-12*$^+$ and the other genes (Table 9). It appears that the upstream region of the *cyt-12*$^+$ gene contains sequences related to the upstream regions of other structural genes encoding mitochondrial products. However, the role of these short sequences in gene regulation, if any, is unknown.

Cloning the *Cyt-12-1* Mutant Gene

DNA purified from the *cyt-12-1* strain was separately digested with the restriction enzymes BamHI, EcoRI, HindIII, and a combination of both HindIII and PstI. Following agarose electrophoresis, these DNAs were examined by southern analysis, utilizing the 3.9 kb HindIII-PstI *cyt-12*$^+$ fragment as probe (Fig. 16). The *cyt-12-1* strain contained a HindIII/PstI fragment that comigrates with the 3.9 kb wild-type fragment (Fig 16). The results also suggested that *cyt-12-1* contains a single copy of the cytochrome *c* gene in agreement with the work of Stuart *et al.*(1987) who reached a similiar conclusion from southern analysis of different digests of *N.crassa* wild-type (*74A*) DNA.

A large scale HindIII/PstI digest of *cyt-12-1* nuclear DNA was fractionated on a continuous sucrose gradient. A small aliquot of each fraction was analysed by gel electrophoresis to determine which fractions contained genomic DNA in the range of 2 to 5 kb in length. The appropriate fractions were pooled, "shotgun" cloned into the plasmid pUC19 that had also been digested with HindIII and PstI, and the ligation products transformed into *E.coli* strain JM83. The partial *cyt-12-1* genomic library (approximately 1500 white colonies) was patched onto biodyne membrane and examined as described in Materials and Methods. The colonies were probed with ^{32}P-labelled 3.9 kb *cyt-12*$^{+}$ HindIII-PstI fragment isolated from the pSP8 clone. Three colonies appeared positive and were chosen for further analysis. Plasmid DNA isolated from these clones was digested with the enzymes HindIII and PstI and analysed by agarose gel electrophoresis (not shown). Only one clone, designated pDB1, contained an insert that comigrated with the wild-type version on the agarose gel, while the other two clones (pDB2 and pDB3) contained inserts smaller than expected and were probably detected as a result of non-specific hybridization in the screening procedure. The clone designated pDB1 (see Figure 7 for partial restriction map), was selected for large scale isolation of plasmid DNA and sequence analysis of the *cyt-12-1* mutation.

DNA Sequence Analysis of the *Cyt-12-1* Mutant Allele

The nature of the *cyt-12-1* mutation was determined by subcloning three *cyt-12-1* fragments, derived from pDB1, into M13 phage vectors. The partial restriction map and DNA sequencing strategy of the *cyt-12-1* gene is shown in

Figure 17. Cytochrome *c* specific primers, cyt*c*1, cyt*c*2, cyt*c*3, and cyt*c*4; 16 to 18 nucleotides in length, synthesized by Dr. Ken Roy, Department of Microbiology, University of Alberta were used, in addition to the M13 universal primer, to obtain sequence of the *cyt-12-1* mutant allele (Fig. 17). The DNA sequence of the *cyt-12-1* allele was compared to the *cyt-12*$^+$ gene and found to contain two single nucleotide changes, a deletion of a G at position -363 and a missense mutation (wild-type G changed to an A residue) at position 579 (Fig.18). Single stranded sequence of the *cyt-12-1* gene was obtained except in the region of the missense mutation, in which both strands were sequenced. The missense mutation of the *cyt-12-1* allele caused the codon at position 10 to encode an aspartic acid instead of the glycine observed in the *cyt-12*$^+$ gene (Table 10).

The Spring 1989 version of Microgenie™ databank (Beckman Inc., 1989) was found to contain the amino-acid sequence of 84 cytochrome *c* proteins from various species. The cytochrome *c* proteins were aligned according to homology and it was observed that the glycine corresponding to that at position 10 of the Neurospora sequence, was conserved in all the listed cytochrome *c* proteins. Thus, it seemed that the missense mutation of the *cyt-12-1* allele was the mutation represented by the mutant phenotype.

However, it was conceivable that the single base deletion at position -363 affected normal expression of the cytochrome *c* mutant allele. DNA sequence analysis of the *cyt-12*$^+$ gene revealed that the restriction enzyme BamHI cuts the gene at position -178. Earlier transformation experiments using BamHI digests of pSV50-5324 gave a frequency of transformants that was intermediate between the numbers obtained with those enzymes that cut the gene and those that did not cut the gene (see Table 5). Therefore, the possiblity existed that the deletion at -363 might decrease transcription of the *cyt-12-1* allele. Therefore, further studies were undertaken to analyse *cyt-12*$^+$ and *cyt-12-1* transcripts.

Subcloning the Benomyl-resistant Beta-tubulin Gene

In order to quantify cytochrome *c* transript levels (below), another probe was necessary that would also detect mRNAs whose levels are fairly constant in both wild-type and cyt-12-1 cells. One such probe would be from the benomyl-resistant beta-tubulin gene (Bn^R; Orbach *et al.*, 1986), which was previously used as a control in *N.crassa* transformation experiments (discussed earlier). During this study the 2.58 kb SalI-SalI fragment of pSV50 (Fig. 4), containing the Bn^R gene, could not readily be purified from our lab stocks of pSV50, since they had been contaminated with various deletion derivatives of pSV50 which presumably reflects an instability of the inserted sequences in *E.coli* cells. To obtain a more easily maintained stock and to simplify the purification of this fragment the 2.58 kb SalI fragment isolated from pSV50 was cloned into pUC19. Following transformation of the ligation products into E.coli JM83 and selection on X-gal medium, several white colonies were chosen for further analysis. Plasmid DNA isolated from these clones were digested with SalI and analysed by gel electrophoresis. All eight clones selected, contained SalI inserts that comigrated with the remaining stock of 2.58 kb SalI-SalI fragment. Two clones, designated pBEN1 and pBEN2, probably contain the SalI insert in opposite orientations (Fig.19) as judged by further restriction analysis (not shown).

Northern Analysis

Wild-type and *Cyt-12-1* transcripts were examined by analysing total RNA and poly (A) RNA isolated from these strains. Total RNA was isolated by a phenol-SDS RNA extraction procedure, while poly (A) RNA purification kits utilizing prehydrated oligo-dT cellulose columns were used to enrich total RNA preparations for mRNA. The plasmid pRCYC1, a generous gift of Drs. Walter Neupert and Rosemary Stuart (University of Munich), containing the full-length wild-type (*74A*) cytochrome *c* cDNA (680bp), was used as a source of cytochrome *c* cDNA fragment for oligo-labelling reactions. Two samples of total RNA and poly (A) RNA, from several RNA preparations of wild-type and *cyt-12-1* strains, were examined by agarose-formaldehyde gel electrophoresis. Half of the RNA samples were studied by northern analysis using ^{32}P-labelled cytochrome *c* cDNA (purified from pRCYC1; Fig. 20A) and the other half analysed using the ^{32}P-labelled 2.58 kb SalI fragment containing the Bn^{R} gene (isolated from pBEN1; Fig. 20B) as probes. Cytochrome *c* and beta-tubulin transcripts both migrated at apparently faster rates in poly (A) RNA samples than in total RNA preparations (Fig.20A and 20B, compare lanes G to J with lanes A to F). In attempt to discover the reasons for this anomaly several aliquots of wild-type and *cyt-12-1* total RNA were analysed by gel electrophoresis. These samples were found to contain various amounts of chromosomal DNA and ribosomal RNA. To test if either the contaminating genomic DNA or the large amounts of rRNA were the source of the problem, various amounts of wild-type total RNA were added to different amounts of wild-type mRNA, analysed by agarose-formaldehyde electrophoresis, and by northern anaylsis using both cytochrome *c* cDNA and beta-tubulin probes (not shown). The evidence regarding this migration rate dilemma was not clear. Therefore, cytochrome *c* transcript lengths could only be compared within total RNA or within poly (A) RNA isolates. *Cyt-12-1* transcripts appeared to be *ca*. 700 nucleotides in length and comigrated with wild-type cytochrome *c* transcripts (Fig.20A, compare lanes G and H with lanes I and J).

Although, no attempt was made to quantify the transcript levels exactly and the quality of the RNAs is rather poor, it appears that the two strains contain roughly similiar levels of both cytochrome *c* mRNA as judged by corresponding levels of beta-tubulin mRNA (Fig.20). However, two to three fold differences can not be ruled out. Thus, it is likely that the single base deletion at -363 in the upstream region of the *cyt-12-1* gene does not have a gross affect on cytochrome *c* transcript levels in the *cyt-12-1* mutant strain. The specific activities of the beta-tubulin and cytochrome *c* cDNA probes used for northern analysis were approximately equal. When the level of cytochrome *c* mRNA is compared to the beta-tubulin transcript levels (Fig.20A, compare lanes A to F to the same lanes of Fig.20B), it appears that the cytochrome *c* gene is expressed about as highly as the beta-tubulin gene, suggesting it is also a highly expressed mRNA. This is in agreement with the codon usage criteria of highly expressed genes.

Apocytochrome *c* and Holocytochrome *c* Protein Analysis

Since *cyt-12-1* appeared to contain cytochrome *c* mRNA and yet contained no spectrally detectable cytochrome *c*, it was of interest to determine if *cyt-12-1* mRNA could be used to program an *in vitro* translation system to produce apocytochrome *c*.

Wild-type (NCN10) and *cyt-12-1* mRNA were used to program rabbit reticulocyte *in vitro* translation reactions, utilizing ^{35}S-labelled methionine as the radioisotope. The translation products (1.5 X 10^6 cpm from *cyt-12-1*; 4.5 X 10^6 cpm from wild-type reactions) were examined for the presence of immunoprecipitable apocytochrome *c*. The cytochrome *c* specific antibodies were generous gifts from Drs. Walter Neupert, Rosemary Stuart, and Christoph Hergersberg (University of Munich). These immunoprecipitates were separated on a 10% discontinuous SDS-polyacrylamide gel. Following electrophoresis, the lanes were cut into gel slices and processed according to Materials and Methods. The cpm per gel slice from the two immunoprecipitations (wild-type and *cyt-12-1*) were plotted (Fig.21 and 22, respectively). All gel slicing was performed backwards to normal convention (*i.e.* slice zero corresponds to the bottom of the gel). Both strains gave rise to immunologically detectable apocytochrome *c* (Fig.21 and 22). Apocytochrome *c* dimers were observed in both immunoprecipitations even though the final concentrations of SDS and beta-mercaptoethanol were increased (from 2.5% to 5% and 2.5% to 7%, respectively) during the cracking step.

The total number of counts of *cyt-12-1* apocytochrome *c* (1.8 X 10^4 cpm) was approximately the same as the total number of counts of wild-type immunoprecipitated apocytochrome *c* (2.2 X 10^4 cpm). The total number counts of the wild-type *in vitro* translation mixture used for the immunoprecipitation was 3 times more than the amount used of *cyt-12-1 in vitro* translation products (4.5 X 10^6 cpm as compared to 1.5 X 10^6 cpm). Thus, at least from this single determination, it appears that *cyt-12-1* apocytochrome *c* is expressed approximately 3 times higher than that of the wild-type strain.

In an attempt to determine if holocytochrome *c* could be detected immunologically in the mitochondria of *cyt-12-1*, wild-type (NCN10) and *cyt-12-1* strains were labelled *in vivo* using ^{35}S-methionine and ^{3}H-leucine, respectively. Mitochondria were isolated and solubilized and holocytochrome *c* specific antisera were used to separate holocytochrome *c* from other *in vivo* translation products. The holocytochrome *c* immunoprecipitates from ^{35}S-labelled [wild-type; 10^5 cpm] and ^{3}H-labelled *cyt-12-1* mitochondrial proteins [10^6 cpm] were electrophoresed on a 10% discontinuous SDS-polyacrylamide gel and analysed by fluorography (Fig.23). Holocytochrome *c* protein (approximately 14 kdal) was observed in wild-type translation products (Fig.23, lanes B and C), but no holocytochrome *c* was observed in *cyt-12-1* (Fig.23, lane A). Even longer exposures (up to 96 hours) to compensate for the fact that ^{3}H is not as easily detected as ^{35}S failed to detect any immunoprecipitable *cyt-12-1* holocytochrome c. Since it was conceivable that the *cyt-12-1* holocytochrome *c* immunoprecipitates may have been lost during the washing procedure, the levels of holocytochrome *c* of the two strains were also analysed, by mixing differentially labelled translation products from the wild-type strain and *cyt-12-1*. Holocytochrome *c* immunoprecipitates from the mixture of solubilized ^{35}S-labelled [wild-type; 5 x 10^6 cpm] and ^{3}H-labelled *cyt-12-1* [1.2 x 10^7 cpm] mitochrondria were analysed by polyacrylamide gel electrophoresis (as above). After electrophoresis, the lane was sliced into 1.1 mm gel slices and processed as described in Materials and Methods. The cpm per gel slice of ^{3}H and ^{35}S were determined and were plotted after correcting for spillover of ^{35}S counts into the ^{3}H channel (Fig.24). This coimmunoprecipitation experiment showed that *cyt-12-1* contains very little, if any, immunologically detectable holocytochrome *c* when compared to the background.

IV. Discussion

In contrast to other precursor proteins targeted and imported into mitochondria, cytochrome *c* does not utilize the "classical" import machinery. Thus, the biogenesis of cytochrome c differs in many respects from that of other precursor proteins destined to mitochondria. Although many studies have shed light upon the biogenesis of cytochrome c, many regulatory questions still remain unanswered. In attempt to investigate the regulatory mechanisms of cytochrome *c* biogenesis in *Neurospora crassa*, two cytochrome *c* deficient mutants, *cyc-1-1* and subsequently *cyt-12-1*, were analysed in this study.

A wild-type (*74A*) *N.crassa* genomic library constructed in the cosmid vector pSV50, containing the benomyl resistant beta-tubulin gene as a dominant selectable marker, was screened using sib selection for its ability to rescue the slow growth character of the mutant *cyc-1-1* on media containing benomyl. A cosmid clone, designated pSV50-5324, was isolated that rescues the slow growth phenotype of *cyc-1-1*. During the course of sub-cloning the *cyc-1*$^+$ gene from pSV50-5324, another cosmid (pSV50-4333) was isolated from the same genomic library by others in our laboratory, that rescues the slow-growth character of *cyt-12-1*. Transformation results indicated that a 3.9 kb HindIII-PstI fragment from pSV50-4333, contained the *cyt-12*$^+$ gene. This fragment was subsequently cloned into pUC19, and designated pSP8.

Since *cyc-1-1* and *cyt-12-1*, have similiar cytochrome spectra (completely lack cytochrome *c* and partially deficient of cytochrome aa_3) when grown at 37°C and 25°C, respectively (see Introduction), experiments were undertaken to investigate the possibility that the mutants might be allelic. Complementation analysis of the two mutant strains revealed that *cyc-1-1* and *cyt-12-1* are in fact allelic mutations. Furthermore, the analysis of several transformants from reciprocal transformations indicated that pSV50-4333, pSV50-5324, or pSP8 can restore cytochrome *c* in either strain.

The observation that either cosmid digested with various restriction enzymes gave identical transformation results with respect to destroying the transforming ability of the wild-type gene, suggested that pSV50-4333 and pSV50-5324 might contain the same transforming gene. To determine if this was in fact the case, both cosmids were separately digested with various combinations of restriction endonucleases, and analysed by agarose gel electrophoresis and by southern analysis. Examination of the restriction patterns revealed that many fragments of pSV50-5324 digests comigrated with fragments of identical digests of pSV50-4333, while southern data proved that pSV50-5324 contains the *cyt-12*$^+$ gene. Further analysis revealed that the minor restriction pattern differences observed between digests of pSV50-4333 and pSV50-5324 were the result of cloning different lengths of flanking genomic sequence surrounding the *cyt-12*$^+$ gene. Later it was concluded that the proximity of pSV50 vector sequences to the *cyt*-12$^+$ gene in pSV50-5324 is most likely responsible for the restriction map differences seen between the two cosmids.

The *cyt-12*$^+$ gene subcloned from pSV50-4333 into pUC19 (termed pSP8) was used for DNA sequence analysis. The protein sequence deduced from the DNA sequence corresponded to the previously determined amino-acid sequence of *N.crassa* cytochrome *c* (Heller and Smith, 1966; Lederer and Simon, 1974), while the coding sequence matched the DNA sequence of a wild-type *N.crassa* cytochrome *c* cDNA (Stuart *et al.*, 1987).

Sequence analysis of the wild-type cytochrome c gene revealed the presence of two introns, one intron located near the amino terminus (intron 1; interrupts codon 6) and the other located near the carboxyl terminus (intron 2; interrupts codon 101) (Stuart *et al.*, 1987; Stuart, 1989). Interestingly, all other cytochrome *c* genes analysed to date, such as rat (Scapulla *et al.*, 1981), chicken (Limbach and Wu, 1983), and mouse (Limbach and Wu, 1985a), contain only one intron located near the amino-terminus (Stuart *et al.*, 1987). Sequence analysis of these three cytochrome *c* genes revealed that the location of the intervening sequences are conserved between codons 55 and 56, and that the introns appear to be of relatively equal lengths (Limbach and Wu, 1985a; Stuart *et al.*, 1987; Stuart, 1989). There seems to be no obvious connection between the position of the intron of these cytochrome *c* genes with the locations of the two introns of *N.crassa* cytochrome *c*.

DNA sequence analysis of the *cyc-1-1* mutant allele, revealed that the intron located near the carboxyl terminus is not excised giving rise to mutant apocytochrome *c* 19 amino acids longer than the wild-type apo-protein (Stuart *et al.*, 1987). It was determined that *cyc-1-1* apocytochrome *c* is not imported into mitochondria *in vitro* or *in vivo* (Stuart, 1989). Even though these studies can not conclude if a "targeting signal" is located within the carboxyl terminus of cytochrome c or if the additional residues simply alter the overall conformation of the apo-protein sufficiently to prevent binding and/or targeting (Stuart et al., 1987), they at least demonstrate the functional importance of the correct excision of the intron located near the carboxyl terminus of *N.crassa* cytochrome *c*. The analysis of both *N.crassa* cytochrome *c* introns revealed that the donor splice sequences, splice acceptor sequences, and the branch site sequences of each intron match the consensus sequences derived from the analysis of many filamentous fungi genes (Ballance, 1986).

Normally, *Neurospora* sphaeroplasts cotransformed with restriction endonuclease digested cosmid DNA and intact pSV50 yield clear results. Restriction enzymes that cut the transforming gene should give no or very few fast-growing transformants, while enzymes that do not cut the wild-type gene should yield many fast-growing colonies. DNA sequence analysis revealed that the restriction enzymes that yielded very few, if any, fast-growing transformants were found to have recognition sites in the *cyt-12* gene, while those restriction enzymes that gave many fast-growing transformants do not have sites within the *N.crassa* cytochrome *c* gene, as expected. However, the intermediate transformation results of *cyc-1-1* sphaeroplasts co-transformed with pSV50 and BamHI digested pSV50-5324, suggested that a BamHI restriction site might be located between a positive acting regulatory region and the transcription initiation site of the *N.crassa* cytochrome *c* gene. This finding was supported by DNA sequence analysis of the *cyt-12*$^+$ gene, which revealed that the enzyme BamHI cuts the cytochrome *c* upstream region at -174.

The sequence upstream of the *cyt-12* gene was compared to the upstream activation sequences (UAS) of several yeast genes, known to respond to catabolite repression and intracellular levels of heme. Two potential upstream regions of the *cyt-12* gene were detected that contain some similiarity to the yeast UAS sites. However, no UAS have yet been shown to exist in *N.crassa* by functional analysis.

During the course of cloning the *cyt-12-1* allele, *cyt-12-1* DNA was digested with several restriction enzymes. It was revealed that *cyt-12-1* contained a 3.9 kb HindIII/PstI fragment that comigrated with the corresponding wild-type fragment. These results also showed that *cyt-12-1* contains only one copy of the cytochrome *c* gene and is, therefore, similiar to wild-type strain (*74A*) in this regard (Stuart *et* *al.*, 1987). Previous studies revealed that chicken (Limbach and Wu, 1983) and *Schizosaccharomyces pombe* (Russell and Hall, 1982) also contain only one cytochrome *c* gene per haploid genome.

Sequence analysis of the *cyt-12-1* mutant allele when compared to the wild-type cytochrome *c* sequence, revealed two mutations, a deletion of a G residue at -363 and a transition (G to an A residue) at +579. The G/C to A/T transition at position 579 causes the codon (amimo acid position 10 of the *N.crassa* protein) that normally encodes a glycine residue to be replaced by an aspartic acid. Interestingly, homology studies revealed that all wild-type cytochrome *c* proteins from 84 species contain a glycine at the position that corresponds to position 10 of the *Neurospora* protein. This finding suggested that the amino acid replacement of the *cyt-12-1* allele is most likely responsible for the mutant phenotype.

Many amino-acid replacement mutants have been characterized for iso-1-cytochrome *c* in yeast, which is encoded by the *CYC1* gene (Boss *et al.*, 1981). DNA sequence analysis of many *CYC1* mutants revealed four yeast cytochrome c mutants, *cyc1-6*, *cyc1-523*, *cyc1-570*, and *cyc1-581*, that have the glycine corresponding to codon 10 of the *N.crassa* protein replaced by an aspartic acid, similiar to that of *cyt-12-1* (Hampsey *et al.*, 1986). Spectral analysis of these mutants showed that they contain variable amounts of iso-1-cytochrome *c* (5 to 50% of the wild-type levels), but in all cases the replacements abolish function or synthesis of iso-1-cytochrome *c* (Hampsey *et al.*, 1986). Crystallography studies of tuna apo- and holocytochrome *c* (Takano and Dickerson, 1981a, 1981b), horse holocytochrome *c* (Dickerson *et al.*, 1971), bonito apo- and holocytochrome *c* (Ashida *et al.*, 1971, 1973; Tanaka *et al.*, 1975; Matsuura *et al.*, 1979), and rice holocytochrome *c* (Ochi *et al.*, 1983) all revealed that glycine-10 is located within alpha-helix (running from residues 6 to 17) and occurs within a region where there is no room to accommodate a side chain (Hampsey *et al.*, 1986). Thus, it seems likely that the amino acid replacement of glycine at position 10 of the *N.crassa* cytochrome *c* protein by an aspartic acid residue *cyt-12-1* disrupts the structure of the cytochrome *c* backbone resulting in loss of function or synthesis of holocytochrome *c* (discussed below) (Hampsey *et al.*, 1986).

The intermediate transformation results using BamHI digested pSV50-5324, suggested that the deletion at (-363) of *cyt-12-1* could possibly decrease the level of transcription of cytochrome *c* in *cyt-12-1*. Thus, cytochrome c transcripts of a wild-type strain and cyt-12-1 were examined by northern analysis. The *cyt-12-1* cytochrome *c* transcript comigrated with that of the wild-type strain. Even though no attempt was made to quantitate the levels of cytochrome *c* transcripts in *cyt-12-1* relative to the wild-type strain, and despite the fact that the RNAs are of poor quality, it appears that the levels of cytochrome c mRNAs from the two strains are not grossly different. However, two to three fold differences in transcripts levels can not be ruled out. Nonetheless, this finding suggests that the upstream deletion of the *cyt-12-1* allele does not drastically affect transcription of the cytochrome *c* gene in *cyt-12-1*.

Cyt-12-1 and wild-type cytochrome *c* transcript levels were also compared to the levels of beta-tubulin mRNAs, known to be highly expressed (Orbach *et al.*, 1986). The band intensities of the cytochrome *c* and beta-tubulin transcripts within RNA isolates of *cyt-12-1* and wild-type strains, suggest that the cytochrome *c* gene is expressed at a level similiar to that of beta-tubulin. This is in agreement with the codon usage of the *cyt-12* gene, which is similiar to the codon usage observed in highly expressed *N.crassa* genes, such as beta-tubulin (Orbach *et al.*, 1986), and histones (Woudt *et al.*, 1981). By these criteria the *cyt-12* gene could be considered to be expressed at a high level.

Poor quality of the RNAs obtained in this study may be due to the use of the phenol and SDS extraction methods. Towards the end of this study the guanidinium thiocyanate procedure was used to isolate *cyt-12-1* and wild-type RNA. However, both attempts to isolate RNAs from these two strains using the later method resulted in very poor yields.

It was possible that *cyt-12-1* might contain small amounts of cytochrome *c* protein, not detectable by cytochrome spectral analysis. To examine this possibility, wild-type and *cyt-12-1* mitochondria were examined for immunoprecipitable cytochrome *c* by using antiserum specific for *N.crassa* holocytochrome *c*. It was found that *cyt-12-1* mitochondria are almost completely devoid of immunologically detectable holocytochrome c.

In attempt to understand the nature of the cyt-12-1 holocytochrome c deficiency, the ability of *cyt-12-1* apocytochrome c mRNA to be translated in a heterologous system was investigated. Rabbit reticulocyte lysates separately programed with poly(A) RNA from *cyt-12-1* and wild-type cultures both gave rise to immunoprecipitable apocytochrome *c*. Although no conclusions should be based on an experiment that was done only once, it appears that *cyt-12-1* contains the mRNA for apocytochrome *c* at a level about three times higher than that of the wild-type strain. Since the northern data were not of sufficient quality to rule out small differences in amounts of mRNAs in the two strains, it is possible that *cyt-12-1* cytochrome *c* transcripts are expressed approximately three fold higher than of wild-type cells in an attempt to correct for the loss of holocytochrome *c*. Similiarly, northern analysis of the cytochrome *c* deficient mutant *cyc-1-1* revealed that the amount of incorrectly spliced cytochrome *c* transcripts seems to be higher in the mutant than cytochrome *c* mRNAs seen in the wild-type strain (Stuart *et al.*, 1987). Analysis of another cytochrome *c* deficient mutant, *cyt-2-1*, revealed that the mutation affects the coding sequence for cytochrome *c* heme lyase (Nargang *et al.*, 1988; Drygas *et al.*, 1989), which attachs the heme group to apocytochrome *c* (Hennig and Neupert, 1981; Nicholson *et al.*, 1987). Although *cyt-2-1* cytochrome *c* transcripts levels were not directly examined, slightly greater levels of immunoprecipitable apocytochrome *c* were found in rabbit reticulocyte lysates programed with *cyt-2-1* mRNA than that found in rabbit reticulocyte lysates programed with wild-type poly(A) enriched RNA (Nargang *et al.*, 1988). Thus, it appears that *cyc-1-1* and *cyt-2-1*, similiar to *cyt-12-1*, might be overproducing cytochrome *c* mRNA in an attempt to compensate for the loss of functional cytochrome *c*.

The fact that *cyt-12-1* apocytochrome *c* mRNA can be translated and that holocytochrome *c* does not accumulate in the mitochondria of *cyt-12-1*, suggests that mutant *cyt-12-1* apocytochrome *c* is not imported into mitochondria. Since apocytochrome *c* import in *N.crassa* is known to be concommitant with heme attachment (discussed in Introduction), it is difficult to speculate at what point the mutation affects transport and/or heme attachment. Conceivably, *cyt-12-1* apocytochrome *c* could either lack the ability to spontaneously insert into the outer membrane, the ability to be positioned correctly to specifically interact with the specific surface receptor (cytochrome *c* heme lyase), and/or the ability to act as a suitable substrate for heme attachment by cytochrome *c* heme lyase. Conceivably, scatchard analysis of *cyt-12-1* apocytochrome *c* binding to wild-type and *cyt-12-1* mitochondria might reveal the nature of the binding of mutant apocytochrome *c* to *cyt-12-1* mitochondria.

It is interesting that virtually all *Neurospora* mutants lacking cytochromes *aa*$_3$ and/or *b* have abnormally high levels of cytochrome *c*, while cytochrome *c* deficient mutants, such as *cyt-2-1*, *cyc-1-1* and *cyt-12-1*, partially or completely lack cytochrome *aa*$_3$. These results suggest that a complex regulatory system exists to control the expression of cytochromes *aa*$_3$, *b*, and *c* in the electron transport chain.

The observation that the *cyt-2* gene encodes the enzyme cytochrome *c* heme lyase, suggested that it is unlikely that this defect directly affects the production of cytochrome *aa*$_3$ (Nargang *et al.*, 1988). Interestingly, both *cyt-2-1* and the extranuclear mutant [*mi-3*] (characterized by a deficiency of cytochrome *aa*$_3$) can be induced to synthesize cytochrome *aa*$_3$ by growth in medium containing low concentrations of antimycin A, a potent inhibitor of electron transport between cytochrome *b* and cytochrome *c*$_1$ of the electron transport chain (Bertrand and Collins, 1978). It has been determined by immunological methods that *cyt-2-1* and [*mi-3*] contain little, if any, fully assembled cytochrome *c* oxidase, but possesses a high molecular weight form of the mitochondrially encoded subunit 1 of cytochrome *c* oxidase (Bertrand and Werner, 1979; Nargang *et al.*, 1988). These results suggested that unassembled cytochrome *c* oxidase was the result of defective processing of cytochrome *c* oxidase subunit 1

(Bertrand and Werner, 1979). It has been postulated that the missense mutation located in the coding region of the [*mi-3*]*oxi-3* gene, that encodes cytochrome *c* oxidase subunit 1, is most likely responsible for the [*mi-3*] phenotype (Lemire and Nargang, 1986). However, preliminary studies of several *N.crassa* mutants that lack cytochrome aa_3, by others in our laboratory, suggest that almost all such mutants may accumulate the high molecular weight form of subunit 1 observed in [*mi-3*]. Conceivably, the high molecular weight form of subunit 1 may occur in any mutant that is unable to assemble cytochrome *c* oxidase. Thus, since all *Neurospora* mutants that lack cytochrome *c*, are at least partially deficient of cytochrome aa_3 at some temperatures, and since *cyt-2-1* is known to contain the high molecular weight form of subunit 1, it may be that functional cytochrome *c* is necessary for efficient processing or assembly of cytochrome *c* oxidase in *Neurospora*. The degree of the cytochrome aa_3 deficiency could depend on the severity of the cytochrome *c* deficiency. This postulated requirement of cytochrome *c* can apparently be by-passed in certain situations, since *cyb-1-1* (characterized by a deficiency of cytochrome *b*) *cyt-2-1* double mutants contain substantial amounts of cytochromes aa_3 and b, but lack cytochrome *c* (Bertrand and Collins, 1978; Nargang *et al.*, 1988). However, *cyb-1-1*/[*mi-3*] double mutants contain substantial amounts of cytochromes *c* and aa_3, but lack cytochrome *b* (Bertrand et al., 1977).

Any model to explain the interactions in this system must account for at least four sometimes conflicting observations. First, a blockage of electron transport in the cytochrome bc_1 region of the electron transport chain by antimycin A or *cyb-1-1* induces the synthesis of cytochrome aa_3 in *cyt-2-1* and [*mi-3*]. Thus, it appears that a blockage of electron transport in the cytochrome bc_1 region may result in increased efficiency of assembly of cytochrome *c* oxidase. Conceivably, the missense mutation in subunit 1 of [*mi-3*] simply decreases the efficiency of cytochrome *c* oxidase assembly and this can be restored by the action of antimycin A or *cyb-1-1*. Second, a severe deficiency of cytochrome *c* in *cyt-2-1*, *cyt-12-1*, and *cyc-1-1* (grown at 37°C) leads to, by an unknown mechanism, partial or complete deficiencies of cytchrome aa_3 (Mitchell *et al.*, 1953; Bertand *et al.*, 1977; Bottorff, D.A. and Nargang, F.E., unpublished results). Third, *cyb-1-1*[*mi-3*] double mutants lack cytochrome *b*, while *cyb-1-1*/*cyt-2-1* double mutants contain normal levels of cytochrome *b*. Fourth, *N.crassa* mutants deficient in cytochrome aa_3 and/or cytochrome *b* contain higher than normal levels of cytochrome *c*. Although it is difficult to say with certainty what these observations mean, it has been postulated that the efficiency of oxidative phosphorylation in the cell may influence the expression of cytochromes *c*,*b*, and aa_3 of the electron transport chain (Nargang et al., 1988). However, this model seems too simplistic to explain all the above observations.

Name Change for *Cyt-12-1*

It is proposed that the cytochrome *c* deficient strain, designated *cyt-12-1*, should be renamed *cyc-1-12*, since it was determined that *cyt-12-1* and *cyc-1-1* are in fact allelic mutations affecting the cytochrome *c* structural gene.

Tables

Table 1. Bacterial Strains.

Strain	**Genotype**	**References**
E.coli JM103	Δ(lac-proAB), supE,thi,strA, sbcB15,endA, [F\`traD36,proAB, lacI^{q}ZΔM15]	Messing, 1983; Yanisch-Perron *et al.*,1985
E.coli JM83	ara,Δ(lac-proAB), rpsK,ϕ80,lacZΔM15	Yanisch-Perron *et al.*,1985

Table 2. *Neurospora crassa* Strains.

Strain	**Genotype**	**Source**	**Reference**
74-OR23-1A (*74A*)	A, standard laboratory wild-type	H. Bertrand	
NCN 10	A, nic-1, al-2	H. Bertrand	
NCN 82	cyt-12-1, pan-2	H. Bertrand	Bertrand *et al.*, 1977
NCN 104	cyc-1-1, nic-1, al-?	H. Bertrand	Pittenger and West, 1979

Table 3. Plasmid and Cosmid Cloning Vectors.

Vector	**Antibiotic Resistances**	**References**
pBR322	Ap^R,Tc^R	Bolivar *et al.*, 1977
pUC19	Ap^R	Yanisch-Perron *et al.*,1986
pSV50	Ap^R,Bn^R	Vollmer and Yanofsky, 1986

Table 4. Recombinant Plasmids.

Plasmid	**Description**
pRCYC[1]	*74A* cytochrome *c* cDNA in PstI site of pBR322
pSV50-4333[2]	Genomic version of *74A* cytochrome *c* gene in pSV50 isolated from *74A* cosmid library by rescue of the *cyt-12-1* phenotype
pSV50-5324[3]	Genomic version of *74A* cytochrome *c* gene in pSV50 cosmid isolated from *74A* cosmid library by rescue of the *cyc-1-1* phenotype
pSP8[4]	*74A* cytochrome *c* gene contained on a 3.9 kb Hind III/PstI fragment in pUC19
pBEN1,pBEN2	Benomyl resistant beta-tubulin gene SalI fragment in pUC19
pDB1	NCN82 cytochrome *c* gene contained on a 3.9 kb Hind III/Pst I fragment in pUC19

1. A generous gift from Drs. Walter Neupert and Rosemary Stuart, Institut for Physiologische Chemie, University of Munich.
2. Isolated by Sukran Parmaksizoglu.
3. Isolated independently from pSV50-4333.
4. Sub-cloned from pSV50-4333 by Sukran Parmaksizoglu.

Table 5. Results of Transformations of *cyc-1-1* Sphaeroplasts with digested pSV50-5324

Restriction Enzyme	Transformation Results
BamHI	+
BglII	-
EcoRI	+++
HindIII	+++
KpnI	-
PstI	+++
SalI	-
SphI	+++
SstI	+++
XhoI	-
XmaI	+++

- no transformants
+ few transformants
++ more transformants
+++ many transformants

Table 6. Residue Frequencies of the *Cyt-12*$^+$ Gene

Region	% A	% C	% G	% T	%(A+T)	%(C+G)
Cytochrome c (coding region only)	24.4	31.8	26.5	17.3	41.7	58.3
Cytochrome c gene (plus introns, etc.)	21.5	29.4	24.1	25.1	46.6	53.4

Table 8. Codon Usage of the *N.crassa* Cytochrome *c* Gene

The codon and residue frequencies (in the 3rd position of codons) of the *cyt-12*$^+$ gene are shown. The stop codon (UAA) was included in the calculations.

AMINO ACID	CODON	# OF CODON	FREQ (%)	AA FREQ	AMINO ACID	CODON	# OF CODON	FREQ (%)	AA FREQ
ALA	GCA	0	0		LEU	UUA	0	0	
	GCC	5	4.6			UUG	0	0	6.4
	GCG	0	0						
	GCU	4	3.7	8.3	LYS	AAA	0	0	
						AAG	14	12	12
ARG	AGA	0	0						
	AGG	1	0.9		MET	AUG	3	2.8	2.8
	CGA	0	0						
	CGC	1	0.9		PHE	UUC	6	5.5	
	CGG	0	0			UUU	0	0	5.5
	CGU	1	0.9	2.7					
					PRO	CCA	0	0	
ASN	AAU	0	0			CCC	2	1.8	
	AAC	6	5.5	5.5		CCG	0	0	
						CCU	1	0.9	2.7
ASP	GAU	2	1.8						
	GAC	5	4.6	6.4	SER	UCA	0	0	
						UCC	2	1.8	
CYS	UGU	0	0			UCG	0	0	
	UGC	2	1.8	1.8		UCU	1	0.9	
						AGU	0	0	
GLN	CAA	0	0			AGC	0	0	2.7
	CAG	2	1.8	1.8					
					THR	ACA	0	0	
GLU	GAA	0	0			ACC	7	6.4	
	GAG	6	5.5	5.5		ACG	0	0	
						ACU	2	1.8	8.2
GLY	GGU	5	4.6						
	GGC	10	9.2		TRP	UGG	1	0.9	0.9
	GGA	0	0						
	GGG	0	0	13.8	TYR	UAU	0	0	
						UAC	4	3.7	3.7
HIS	CAU	0	0						
	CAC	2	1.8	1.8	VAL	GUA	0	0	
						GUC	1	0.9	
ILE	AUU	0	0			GUG	1	0.9	
	AUC	5	4.6			GUU	0	0	1.8
	AUA	0	0	4.6					
					STOP	UAA	1	0.9	
LEU	CUA	0	0			UAG	0	0	
	CUC	5	4.6			UGA	0	0	0.9
	CUG	0	0						
	CUU	2	1.8						

Residue Frequencies of the 3rd Codon Position	% A	% C	% G	% T
	0.9	57.7	25.8	15.6

Table 8. Upstream Activation Sequence-like Regions of the *Cyt-12*$^+$ Gene

Possible upstream activation sequences in the *cyt-12*$^+$ are compared to UAS sequences of various yeast genes and potentially analogous regions of the *N.crassa cyt-2-1* gene. The position of the 5' nucleotide of the UAS is relative to the translation start site which has been assigned the position +1.

Gene	Position of the 5' nucleotide	Sequence	Reference
Cyt-12 (N.crassa)	-160	CTCTTGGTTTGCAACTTT	This study
	-146	CTTTTTTGTTCCTACTAT	This study
Cyt-2 (N.crassa)	-238	CTCTTGGCCCATATTTTT	Drygas et al., 1989
	-206	CTCTTCTTGTTATTGAAG	
	-66	CTCTTTTCTGCCCGACAT	
CYC1 (yeast)	-343	CTCTTTGGCCGGGGTTTA	Guarente et al., 1984
	-297	CTCTTTGGCGAGCGTTGG	
CYC3 (yeast)	-232	CTCTTGCGCGGCGTGGGA	Dumont et al., 1987
	-170	CTCTTTTTATCGACTTTT	
CTT1 (yeast)	-437	CTCTCCTGCGTGCTTTCA	Spevak et al., 1986
	-388	CTCTTTTTCAAGGGGATC	

Table 9.

Upstream Regions of *Cyt-12*$^+$ that are Homologous to Upstream Regions of other *Neurospora* Genes.

Regions of similiarity between the upstream region of the *cyt-12*$^+$ gene and other nuclear encoded mitochondrial proteins of *N.crassa*, *cya-4* (Sachs *et al.*,1989), *cyt-18* (Akins and Lambowitz, 1987), *cycph* (Tropshug *et al.*, 1988), *cyt-21* (Kuiper *et al.*, 1988), *mrp-3* (Kreader *et al.*,1989), and *cyt-2-1* (Drygas *et al.*,1989). The position of the 5' nucleotide of the similiarity is relative to the translation start site (+1).

Gene	Position of the 5' nucleotide	Sequence
Cyt-12	-360	CGTTGC-CAGCGAGT
Cya-4	-334	CTGTGA-CAGCGAGT
Cyt-18	-147	TTGCCCAGCAAG
	-33	CGTTG---AGCGAT
Cyt-12	-345	G--TTGCCAGCTGTGAG
Cya-4	-489	G--T-GCCAGCTGTCAA
	-470	GAATTGCCAGGTGAGGG
	-388	TCA--GCCTGCCGTGAG
Cyt-12	-338	GCTGTG-AGCACATTGAG
Cya-4	-268	GGGGTG-AGCAAAGTGGG
Cycph	-107	GCCGTGTAGCAGATT
Cyt-12	-318	AAAAAAAAGT-TGGATCAAGA
Cyt-21	-243	TAAAAAAAGTGTGGCTCATGA
Cya-4	-173	AAAAAAAAGCTCG
Cyt-12	-84	TTCCTGTTC-TCAGTCA
Cya-4	-38	TTCCTAATC-TCTGTCA
Cyt-21	-364	CTCCAGTTCCTCAAT
Cyt-12	-71	TCATC-T--C-AACTTCCA
Cyt-21	-65	CATC-T--CGAACCTCGT
	-27	GCATC-TATC-AACCTCC
Cya-4	-47	TCCTCCT--C-AACTTCCT
Cycph	-26	GGATT-T--C-ATCTTCCA
Cyt-12	-191	CGCCGTGTGGC
Cycph	-108	CGCCGTGTAGC
Cyt-12	-109	CTTTTTCTTCCTCT
Cyt-2	-156	CTTTTTCCACCTCT
Cyt-12	-329	ACATTGA-GGAC
Cyt-2	-52	ACATTCACGGAC
Cyt-12	-210	ACGCTGCAATAC
Cyt-21	-409	ACGCTGCAGTAC
Cyt-12	-280	TGTTCAGCCAGCC
Cya-4	-391	TGGTCAGCCTGCC
Cyt-12	-159	TCTTGGTT-TGCAA
Mrp-3	-113	GCTTGGTT-TGGAA
Cycph	-126	TCTTTGTTGTGCAT
Cyt-12	-276	CA-GCCAGCTTTCTGGAG
Cya-4	-487	GCCAGCTGTCAAGAG
Mrp-3	-223	CAAGCCAGCTT

Table 10.

Amino-Acid Sequence Homology of Several Cytochrome *c* Proteins to *Cyt-12-1* and *Cyt-12*$^+$ Proteins of *N.crassa*

Amino acid sequence of the N-terminus of cytochrome *c* proteins from various species. The missense mutation of *cyt-12-1* (position 10) encodes an asparatic acid instead of a glycine residue, which is observed in that position of 84 cytochrome *c* proteins in the Spring (1989) version of the Microgenie Data Bank.™

Position		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Human, Chimpanzee					NH3	gly	asp	val	glu	lys	gly	lys	lys	ile	phe	val
Horse, Pig					NH3	gly	asp	val	glu	lys	gly	lys	lys	ile	phe	val
Rabbit, Mouse					NH3	gly	asp	val	glu	lys	gly	lys	lys	val	phe	val
Snapping Turtle					NH3	gly	asp	val	glu	lys	gly	lys	lys	ile	phe	val
Chicken, Turkey					NH3	gly	asp	ile	glu	lys	gly	lys	lys	ile	phe	val
Common Snail					NH3	gly	glx	ala	glx	lys	gly	lys	lys	ile	phe	thr
Sunflower	NH3	glu	ala	pro	ala	gly	asp	pro	thr	thr	gly	ala	lys	ile	phe	lys
Spinach	NH3	glu	ala	pro	ala	gly	asn	lys	asp	val	gly	ala	gly	ile	phe	lys
Wheat	NH3	glu	ala	pro	pro	gly	asn	pro	asp	ala	gly	ala	gly	ile	phe	lys
Maize	NH3	glu	ala	pro	pro	gly	asn	pro	lys	ala	gly	glu	lys	ile	phe	lys
Tomato, Potatoe	NH3	glu	ala	pro	pro	gly	asn	pro	lys	ala	gly	glu	lys	ile	phe	lys
Cotton, Castor bean	NH3	glx	ala	pro	pro	gly	asx	val	lys	ala	gly	glu	lys	ile	phe	lys
Cynthia Moth	NH3	gly	val	pro	ala	gly	asn	ala	glu	asn	gly	lys	lys	ile	phe	val
Fruitfly, Hornfly	NH3	gly	val	pro	ala	gly	asp	val	glu	lys	gly	lys	lys	leu	phe	val
Schizosaccharomyces	NH3	pro	tyr	ala	pro	gly	asp	glu	lys	lys	gly	ala	ser	leu	phe	lys
Iso-1 Baker's Yeast	NH3	glu	phe	lys	ala	gly	ser	ala	lys	lys	gly	ala	thr	leu	phe	lys
Iso-2 Baker's Yeast	NH3	gly	phe	lys	pro	gly	ser	ala	lys	lys	gly	ala	thr	leu	phe	lys
N.crassa (wildtype)	NH3	gly	phe	ser	ala	gly	asp	ser	lys	lys	gly	ala	asn	leu	phe	lys
Cyt-12-1 (NCN82)	NH3	gly	phe	ser	ala	gly	asp	ser	lys	lys	$\boxed{\text{asp}}$	ala	asn	leu	phe	lys

Figures

Figure 1.
Electron Transport Chain of *Neurospora* Mitochondria

(References: Jackson and Lightbown, 1958; Lambowitz and Slayman, 1971; Stryer, 1975; Bertrand *et al.*, 1976; Tzagoloff, 1982)

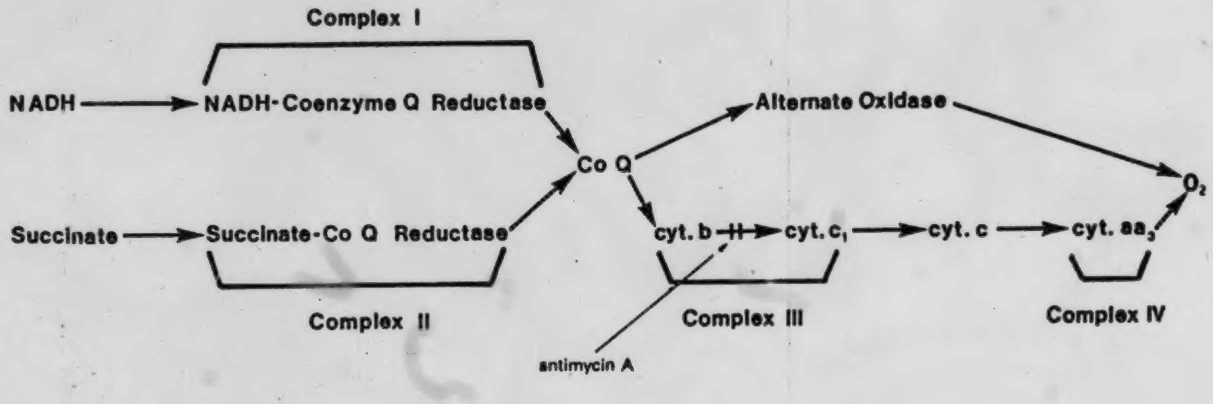

Complex I
NADH
NADH-Coenzyme Q Reductase
Succinate
Succinate-Co Q Reductase
Complex II
Co Q
Alternate Oxidase
O_2
cyt. b
cyt. c_1
cyt. c
cyt. aa_3
Complex III
Complex IV
antimycin A

Figure 2. Cytochrome Spectra of *N.crassa* Strains

Cytochrome spectra of *Neurospora crassa* strains used in this study NCN10 (wild-type), NCN104 (*cyc-1-1*),and NCN82 (*cyt-12-1*) . Cultures were grown at 25°C.

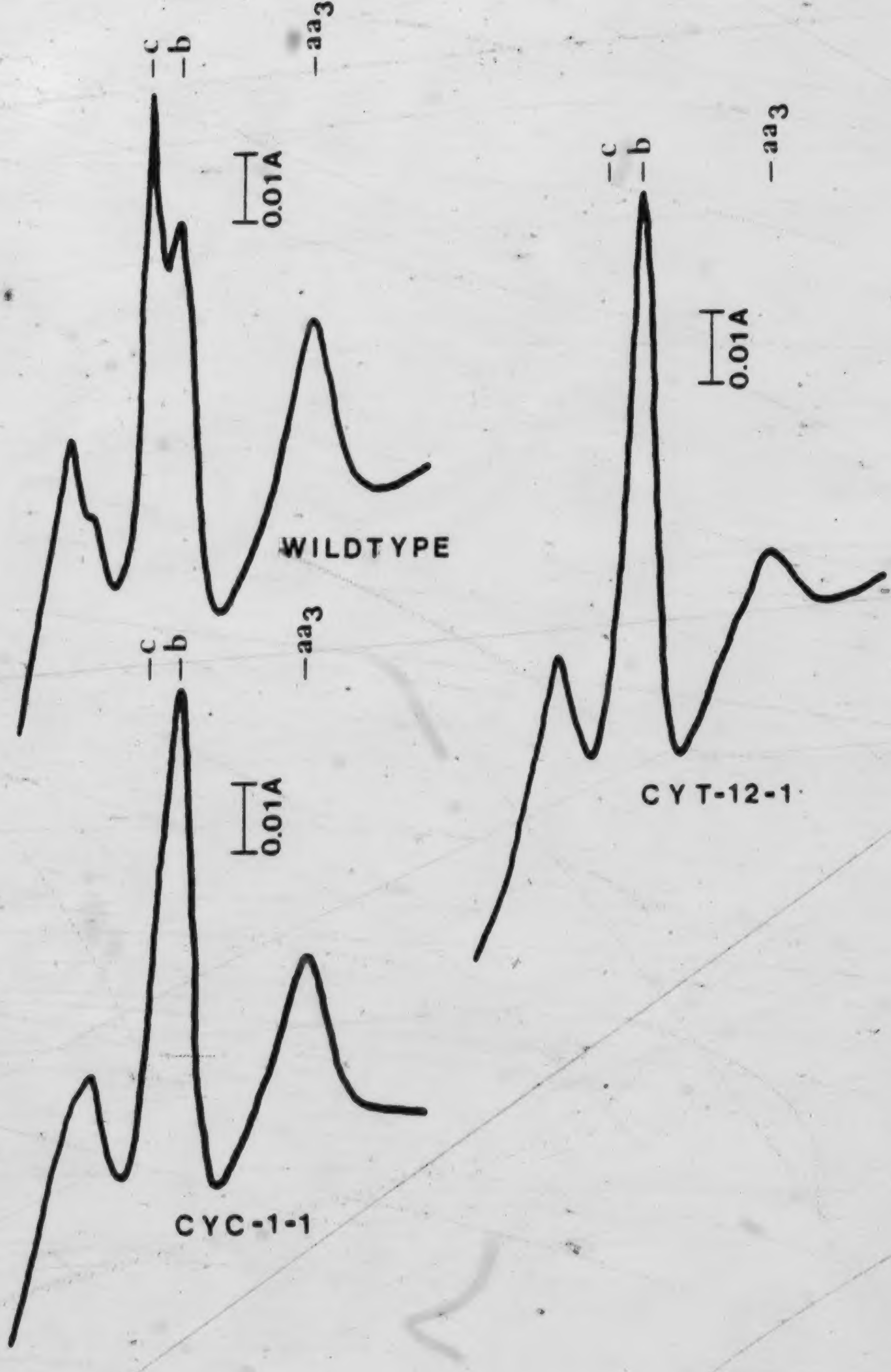

-c
-b
-aa3
0.01A
WILDTYPE
-c
-b
-aa3
0.01A
CYT-12-1
-c
-b
-aa3
0.01A
CYC-1-1

Figure 3.

Import of Cytochrome *c* in *Neurospora crassa*

Apocytochrome c is encoded by a nuclear gene, translated on free polysomes, and released into the cytosol as a soluble protein. Apocytochrome c has the ability to spontaneously insert into the mitochondrial outer membrane, where it binds with high affinity to a specific receptor. It is thought that the enzyme cytochrome c heme lyase (CCHL) is the apocytochrome c receptor and attachs the heme group to partially inserted apocytochrome c. It is believed that the refolding of cytochrome c after heme attachment drives the translocation of the protein across the outer membrane into the intermembrane space.

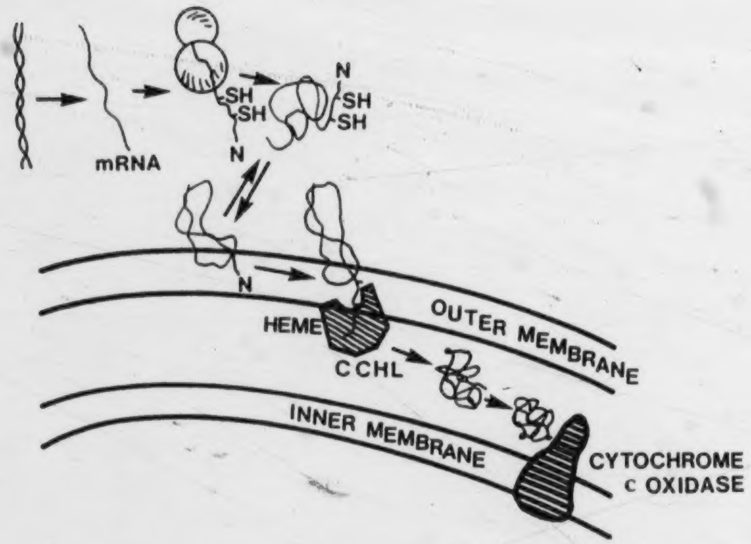

mRNA
SH
SH
N
N
SH
SH
N
HEME
CCHL
OUTER MEMBRANE
INNER MEMBRANE
CYTOCHROME
c OXIDASE

Figure 4.

Partial Restriction Map of pSV50

The partial resriction endonuclease map of the vector pSV50 is shown (adapted from Vollmer and Yanofsky, 1986). The arrows indicate the orientations of the ampicillin resistant (Ap^R) and the benomyl-resistant beta-tubulin (Bn^R) genes. COS = cohesive site region of lambda phage.

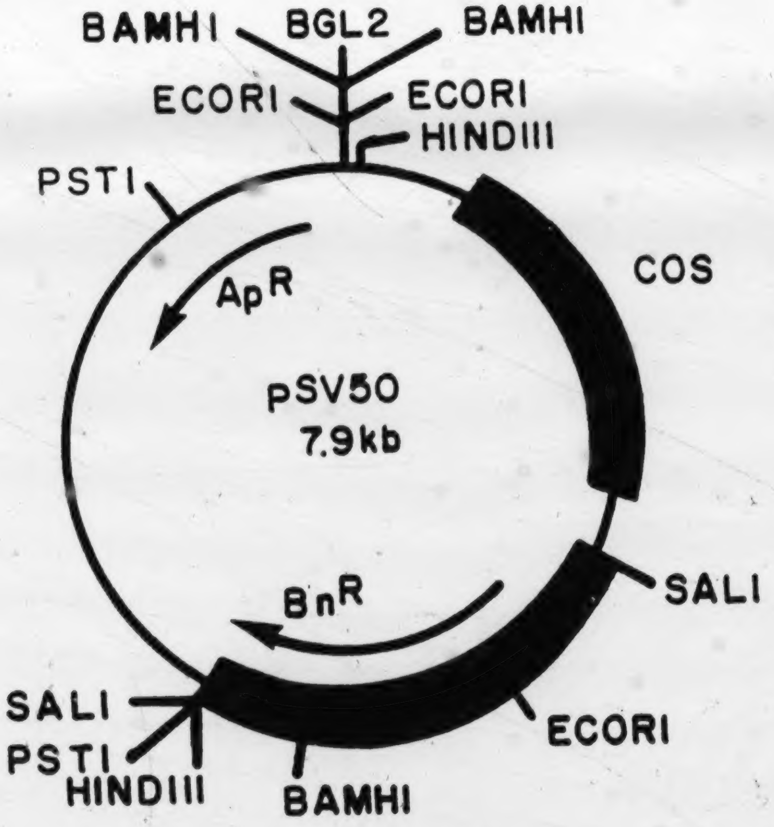
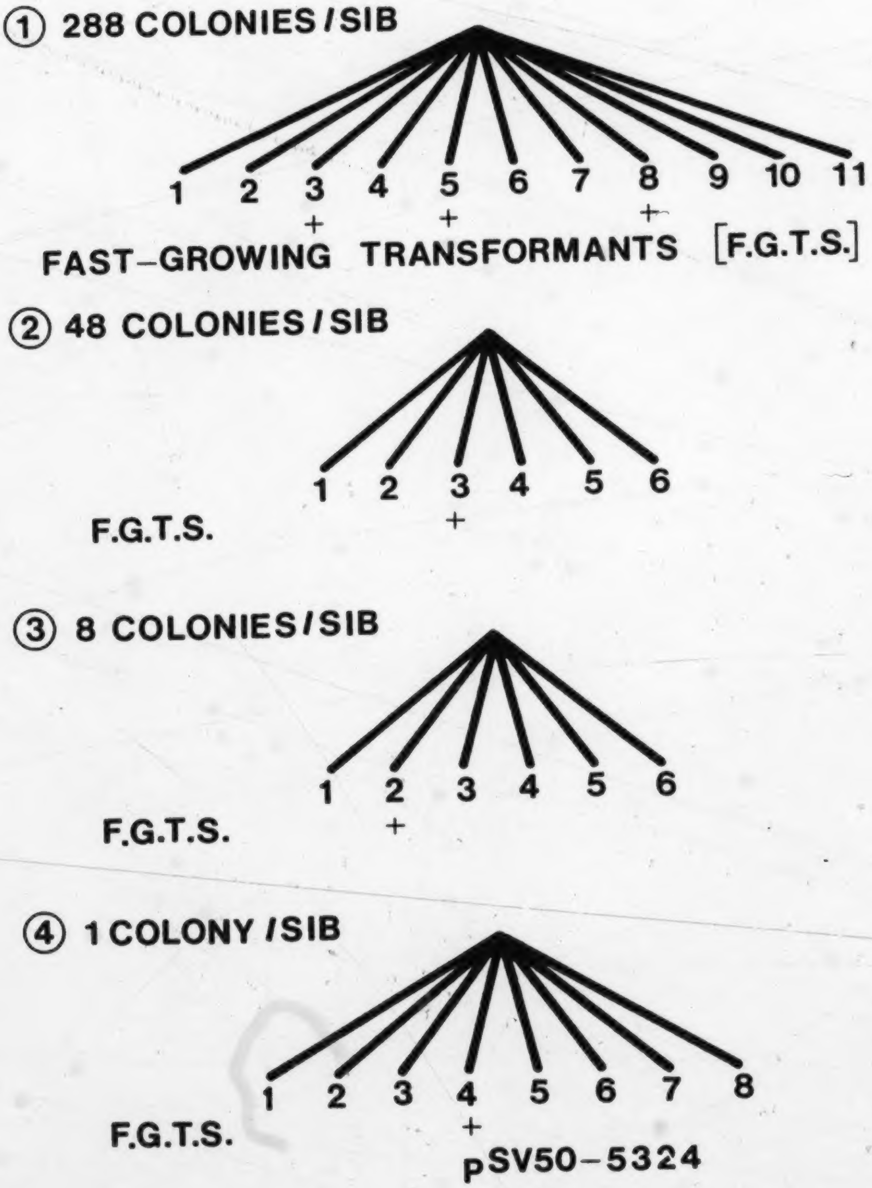

BAMHI
BGL2
BAMHI
ECORI
ECORI
HINDIII
PSTI
COS
Ap^R
pSV50
7.9kb
Bn^R
SALI
SALI
PSTI
HINDIII
BAMHI
ECORI

Figure 5.

Cloning of the *N.crassa Cyc-1*$^+$ by Sib-Selection

Isolation of the cosmid pSV50-5324 from a wild-type (74A) *Neurospora crassa* library by sib selection that rescues the *cyc-1-1* mutant strain.



① 288 COLONIES / SIB

	1	2	3	4	5	6	7	8	9	10	11
FAST–GROWING TRANSFORMANTS [F.G.T.S.]			+		+			+			

② 48 COLONIES / SIB

	1	2	3	4	5	6
F.G.T.S.			+			

③ 8 COLONIES / SIB

	1	2	3	4	5	6
F.G.T.S.		+				

④ 1 COLONY / SIB

	1	2	3	4	5	6	7	8
F.G.T.S.				+				

pSV50–5324

Figure 6.

Agarose Gel of pSV50-4333, pSV50-5324, and pSP8 Digested With Various Restriction Enzymes

Cosmid pSV50-5324, pSV50-4333 or plasmid pSP8 DNAs were cut with different restriction endonucleases and electrophoresed in a 0.8% agarose gel. Lanes A and V contain 1kb ladder marker DNA (BRL, Inc.). Lane B: pSV50-5324 digested with HindIII, PstI, and SalI, Lane C: pSV50-4333 cut with HindIII, PstI, and SalI, Lane D: pSP8 digested with HindIII, PstI, and SalI, Lane E: pUC19 cut with HindIII, PstI, and SalI, Lane F: pSV50-5324 digested with HindIII and PstI, Lane G: pSV50-4333 digested with HindIII and PstI, Lane H: pSP8 digested with HindIII and PstI, Lane I: pUC19 digested with HindIII and PstI, Lane J: pSV50-5324 cut with EcoRI and SalI, Lane K: pSV50-4333 digested with EcoRI and SalI, Lane L: pSV50-5324 digested with SalI, Lane M: pSV50-4333 digested with SalI, Lane N: pSV50-5324 cut with PstI, Lane O: pSV50-4333 cut with PstI, Lane P: pSV50-5324 cut with HindIII, Lane Q: pSV50-4333 cut with HindIII, Lane R: pSV50-5324 digested with EcoRI, Lane S: pSV50-4333 digested with EcoRI, Lane T: pSV50-5324 cut with BamHI, Lane U: pSV50-4333 cut with BamHI. Note that the standard fragments are kilobases in length except for the 506/516 bands, which are base pairs in length.

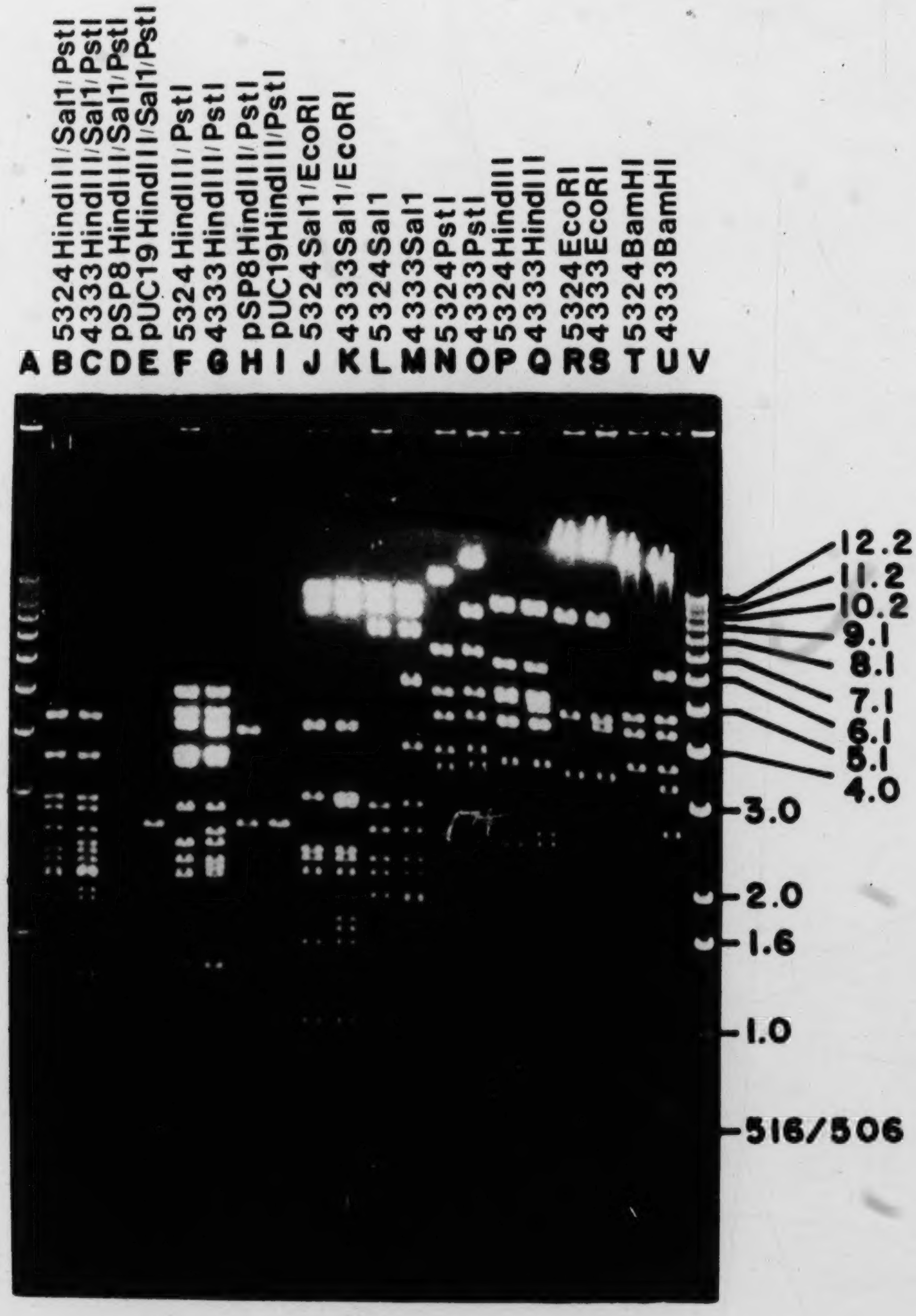
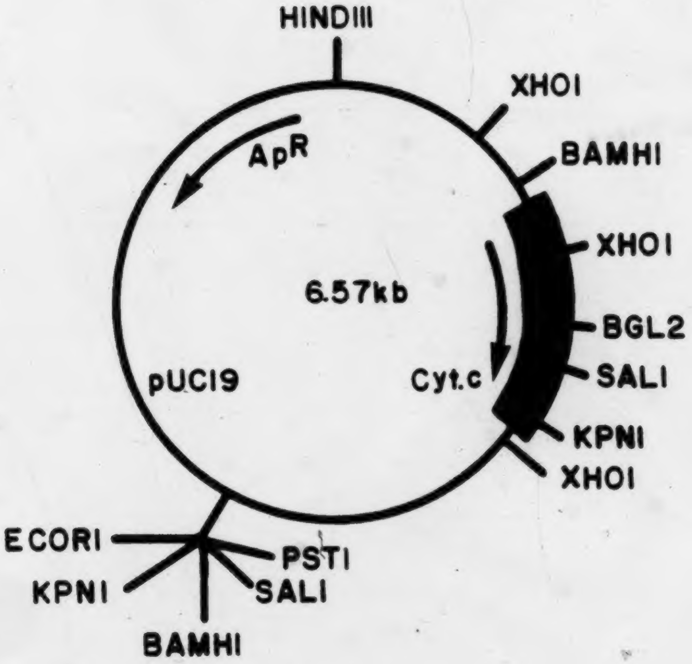

5324 HindIII/Sal1/PstI
4333 HindIII/Sal1/PstI
pSP8 HindIII/Sal1/PstI
pUC19 HindIII/Sal1/PstI
5324 HindIII/PstI
4333 HindIII/PstI
pSP8 HindIII/PstI
pUC19 HindIII/PstI
5324 Sal1/EcoRI
4333 Sal1/EcoRI
5324 Sal1
4333 Sal1
5324 PstI
4333 PstI
5324 HindIII
4333 HindIII
5324 EcoRI
4333 EcoRI
5324 BamHI
4333 BamHI
A B C D E F G H I J K L M N O P Q R S T U V
12.2
11.2
10.2
9.1
8.1
7.1
6.1
5.1
4.0
3.0
2.0
1.6
1.0
516/506

Figure 7.
Partial Restriction Map of the plasmid clones pSP8 and pDB1

The recombinant pUC19 clone pSP8 contains the *cyt-12*$^{+}$ gene. Plasmid pDB1 contains the *cyt-12-1* gene. The arrows indicate the 5' to 3' directions and extent of the ampicillin resistant (Ap^{R}) and the cytochrome *c* genes.


HINDIII
XHOI
BAMHI
XHOI
BGL2
SALI
KPNI
XHOI
ApR
6.57kb
pUC19
Cyt.c
ECORI
KPNI
BAMHI
SALI
PSTI

5

Figure 8.

Cytochrome Spectra of Heterokaryons

Cytochrome spectra of NCN10 (wild-type), NCN82 (*cyt-12-1*), NCN104 (*cyc-1-1*), and a heterokaryon (*cyc-1-1*/*cyt-12-1*).

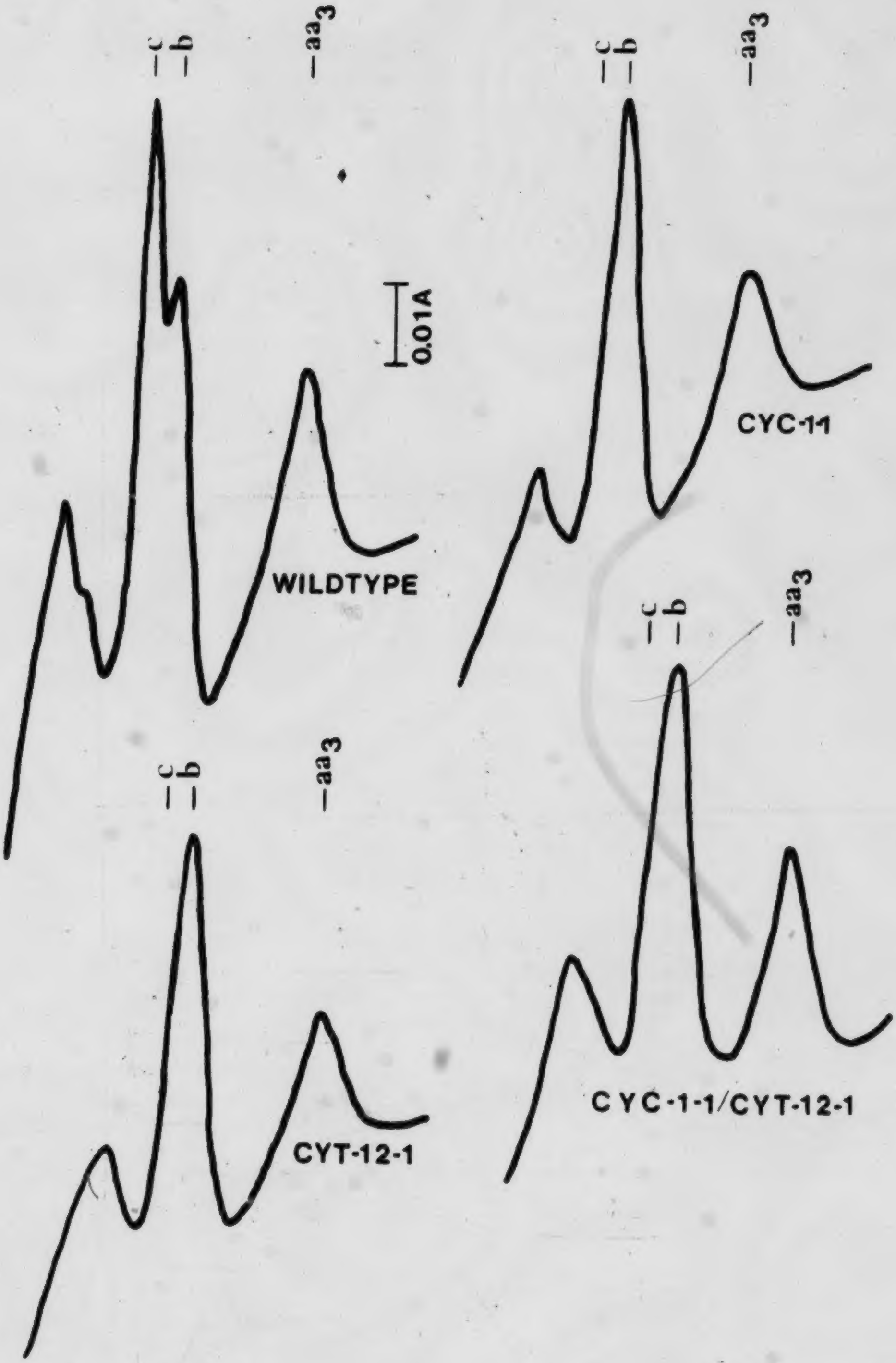
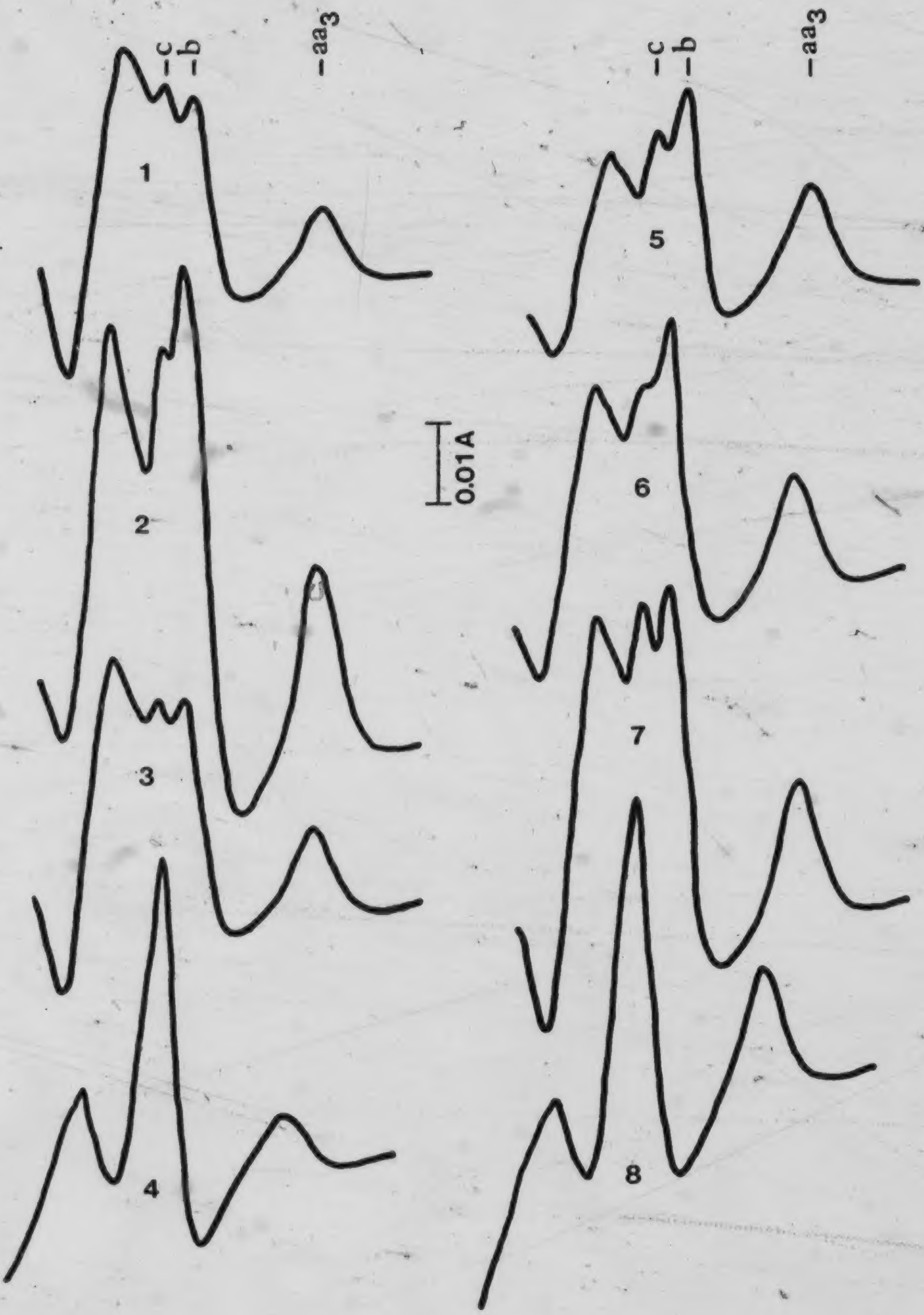

c
b
aa3
0.01A
WILDTYPE
c
b
aa3
CYC-1-1
c
b
aa3
CYT-12-1
c
b
aa3
CYC-1-1/CYT-12-1

Figure 9.

Cytochrome Spectra of Transformants

Cytochrome spectra of *cyc-1-1* and *cyt-12-1* transformants. Spectrum 1: *cyt-12-1* transformed with pSP8, Spectrum 2: *cyt-12-1* transformed with pSV50-4333, Spectrum 3: *cyt-12-1* transformed with pSV50-5324, Spectrum 4: *cyt-12-1*, Spectrum 5: *cyc-1-1* transformed with pSP8, Spectrum 6: *cyc-1-1* transformed with pSV50-4333, Spectrum 7: *cyc-1-1* transformed with pSV50-5324, Spectrum 8: *cyc-1-1*.



–c
–b
$-aa_3$
1
2
3
4
5
6
7
8
0.01A

Figure 10.

Southern Blot Identifying pSV50-5324
as Carrying the *cyt-12*$^+$ gene

Southern analysis of pSV50-4333, pSV50-5324, pUC19, and pSP8 DNA digested with several restriction enzymes. The DNA from an agarose gel (Figure 6) was blotted onto biodyne membrane and probed with ^{32}P-labelled 3.9 kb HindIII-PstI fragment that rescues the mutant strain *cyt-12-1*. Lanes B to U of Figure 10 correspond with the Lanes B to U of Figure 6. The marker used is the 1 kb ladder marker (BRL, Inc.).

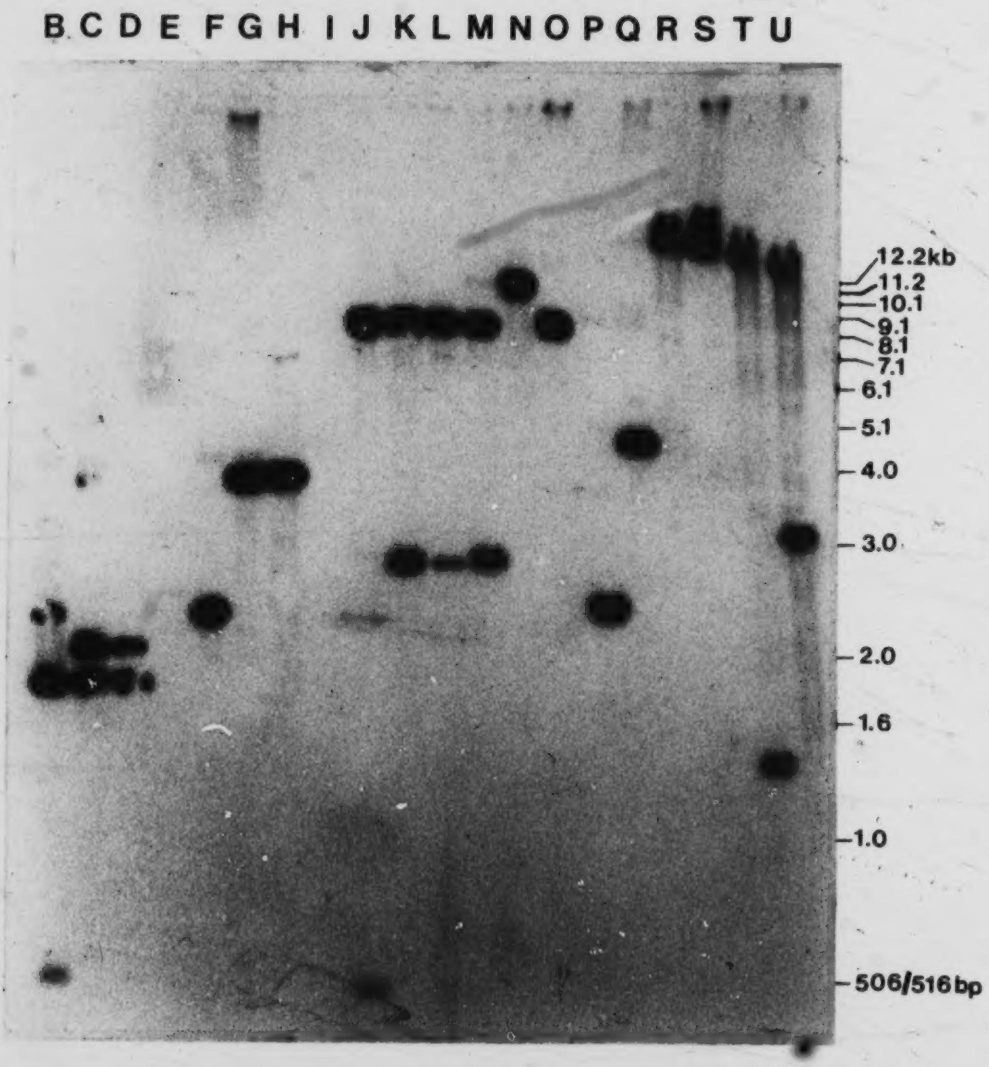

B C D E F G H I J K L M N O P Q R S T U
12.2kb
11.2
10.1
9.1
8.1
7.1
6.1
5.1
4.0
3.0
2.0
1.6
1.0
506/516bp

Figure 11. Agarose Gel of pSV50-4333 and pSV50-5324 Digested With Various Restriction Endonucleases

Cosmid (pSV50-4333 and pSV50-5324) DNA was digested with different restriction endonucleases and electrophoresed in a 0.8% agarose gel. Lane A contains pSV50-5324 cut with SalI, Lane B: pSV50-4333 digested with SalI, Lane C: pSV50-5324 cut with PstI, Lane D: pSV50-4333 digested with PstI, Lane E: pSV50-5324 cut with HindIII, Lane F: pSV50-4333 digested with HindIII, Lane G: pSV50-5324 cut with EcoRI, Lane H: pSV50-5324 digested with BamHI, Lane I: pSV50-4333 cut with EcoRI, Lane J: pSV50-4333 digested with BamHI, Lane K: 1 kb ladder marker DNA (BRL, Inc.)

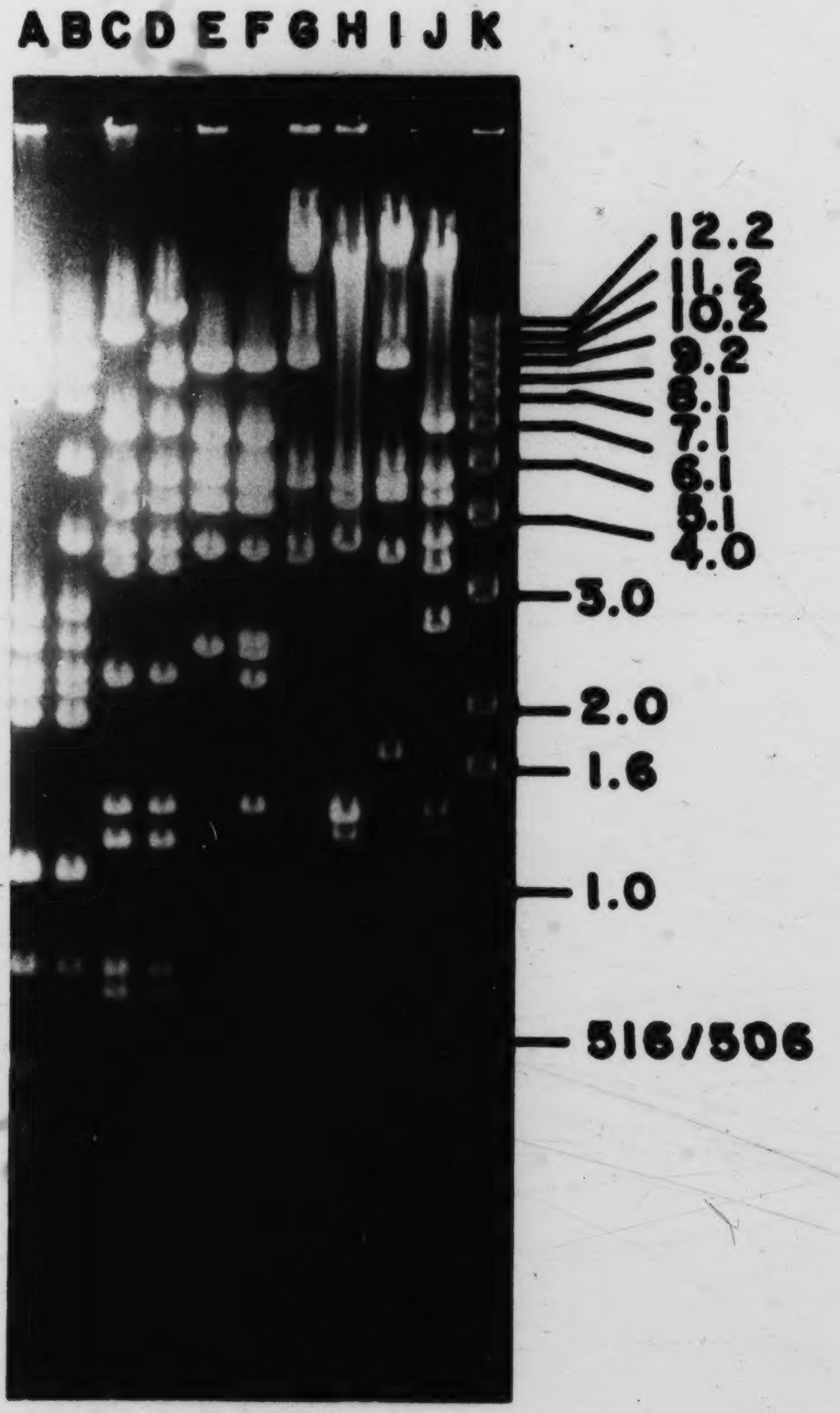

A B C D E F G H I J K
12.2
11.2
10.2
9.2
8.1
7.1
6.1
5.1
4.0
3.0
2.0
1.6
1.0
516/506

Figure 12.

Southern Blot of Cosmid DNA Digested with Different Restriction Enzymes

Southern analysis of cosmid pSV50-4333 and pSV50-5324 DNA cut with various restriction enzymes (Figure 11), blotted onto biodyne membrane, probed with ^{32}P-labelled 1.8 kb HindIII-SalI fragment, stripped, and reprobed with ^{32}P-labelled 2.1 kb SalI-PstI fragment as probes (Figures 12A and 12B, respectively).

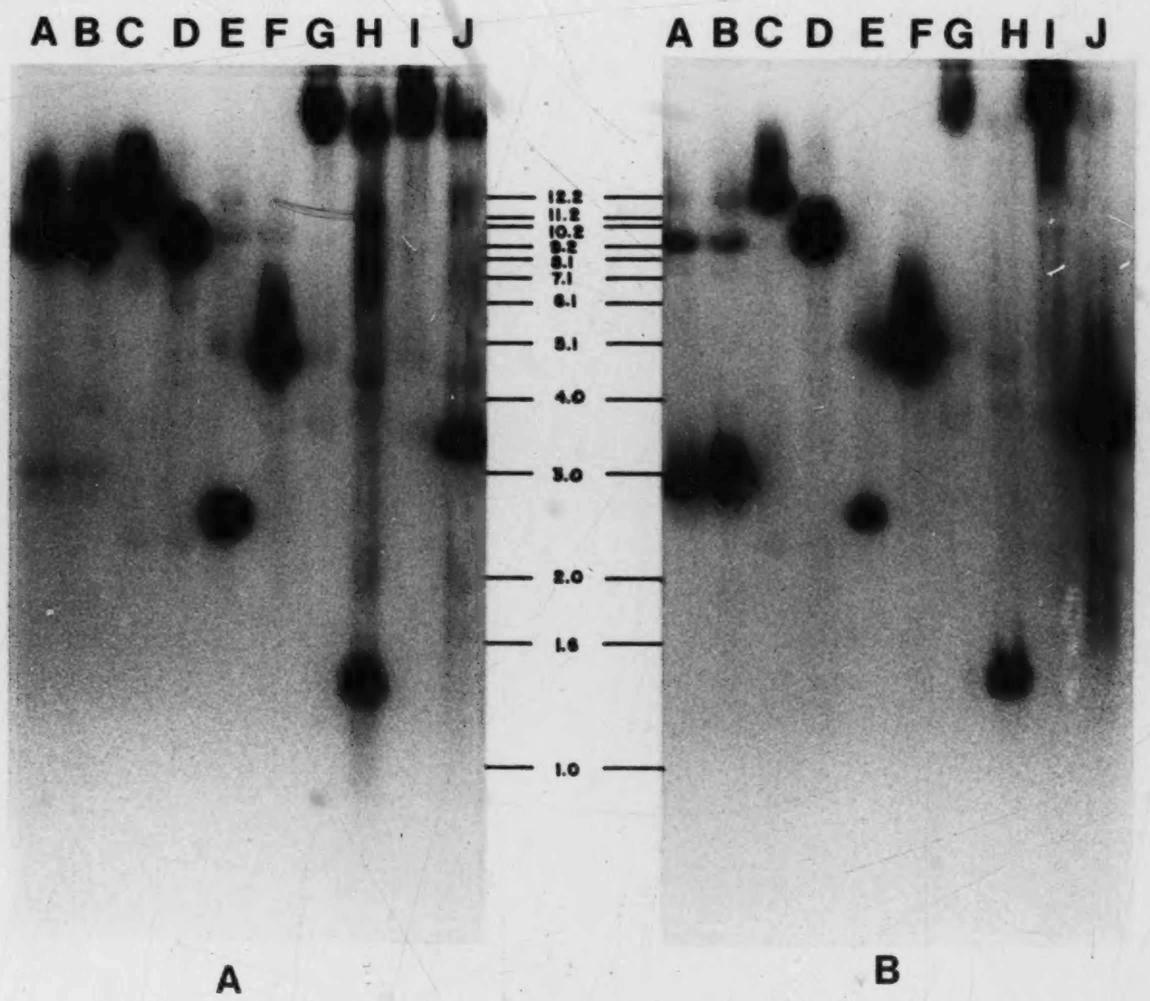

A B C D E F G H I J
A B C D E F G H I J
12.2
11.2
10.2
9.2
8.1
7.1
6.1
5.1
4.0
3.0
2.0
1.6
1.0
A
B

Figure 13. Partial Restriction Maps of the Cosmids, pSV50-4333 and pSV50-5324

Partial restriction maps of pSV50-4333 and pSV50-5324 shows that the different restriction patterns observed between the two cosmids (Fig.6) is due to the different location of the vector pSV50 relative to the *cyt-12*$^{+}$ gene. The location of the cyt-12+ and cyc-1+ gene was determined later by DNA sequence analysis. The heavy lines represent the coding regions and the thin lines represent the non-coding regions.

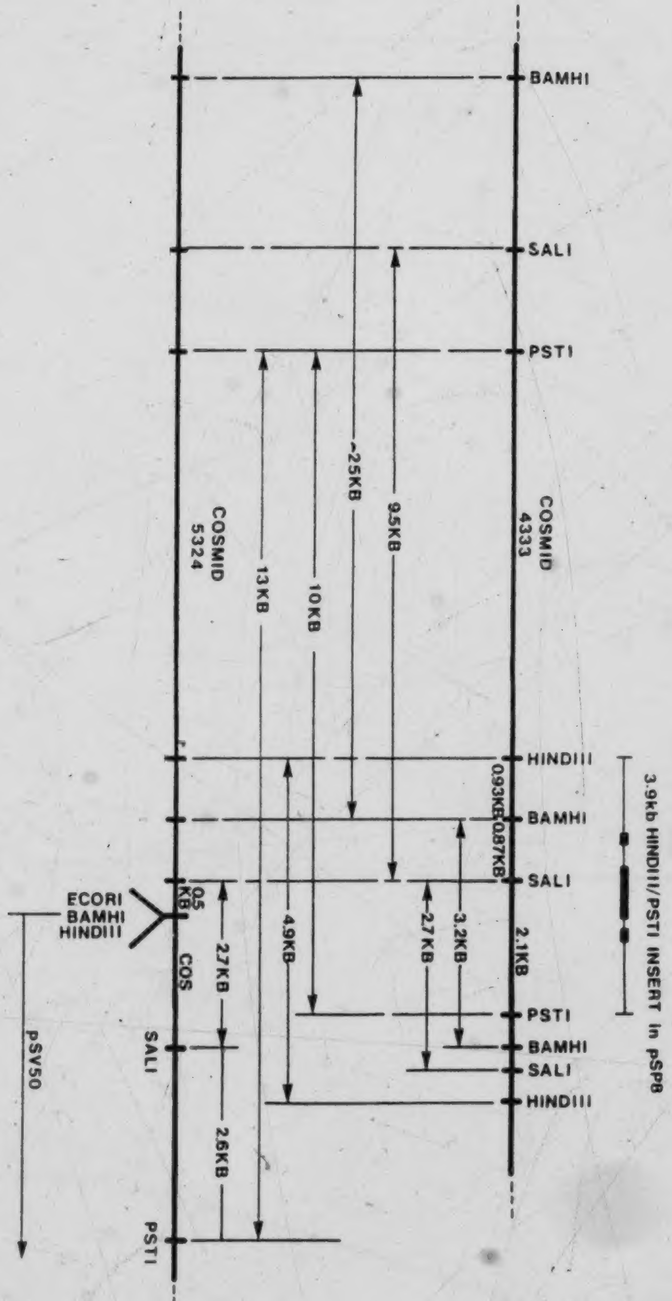

BAMHI
SALI
PSTI
COSMID 433
HINDIII
BAMHI
SALI
PSTI
BAMHI
SALI
HINDIII
3.9kb HINDIII/PSTI INSERT in pSP8
~25KB
9.5KB
0.93KB
0.87KB
3.2KB
2.7KB
2.1KB
COSMID 5324
13KB
10KB
4.9KB
0.5 KB
2.7KB
2.6KB
ECORI
BAMHI
HINDIII
COS
SALI
PSTI
pSV50

Figure 14.
DNA Sequencing Strategy and Partial Restriction Map of the *Cyt-12*$^+$ gene

The arrows indicate the extent and direction of sequences. *Cyt-12*$^+$ fragments from the 3.9 kb HindIII-PstI insert of pSP8, were sub-cloned into M13 phage vectors for DNA sequencing. DNA sequence analysis revealed that the HindIII and the PstI sites are located 5' and 3', respectively, in relation to the *cyt-12*$^+$ gene.

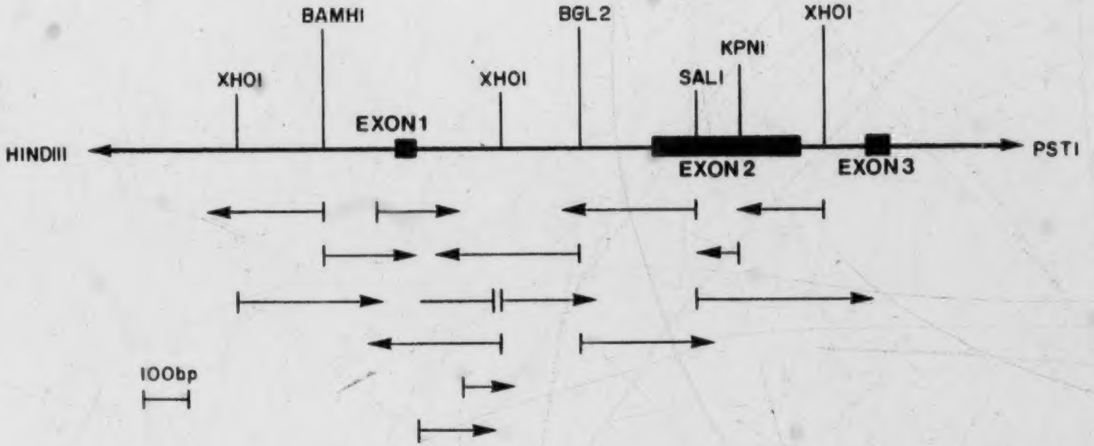

BAMHI
BGL2
XHOI
KPNI
XHOI
XHOI
SALI
EXON1
HINDIII
PSTI
EXON2
EXON3
100bp

Figure 15.

Complete Sequence of the *Cyt-12*$^+$ Gene

The first nucleotide of the coding sequence is designated as +1. The single underlined region represents the nucleotides that are conserved surrounding the ATG codon in *Neurospora* nuclear genes. The double underlined sequences refer to the 5' and 3' intron-exon splice sites. The triple underlined regions are splicing signal sequences for lariat formation that are conserved in fungal genes. The *cyt-12-1* mutant allele contains two single nucleotide changes at positions -363 (▲; deletion of a G residue) and at 579 (■; G is replaced by an A residue).

```
         .         .         .         .         .      -339
CTCGAGATTGCGAATGCCACATGGGATTGGCTGGAGGGCGTTGCCAGCGAGTGGTTGCCA
                                   ▲

         .         .         .         .         .      -279
GCTGTGAGCACATTGAGGACTAAAAAAAAGTTGGATCAAGATCCTCAGCGTGTTGCGGTG

         .         .         .         .         .      -219
TTCAGCCAGCCAGCTTTCTGGAGACAGCGAGCGCGGACGCCGGGGGGAGGCTGGCTTGCA

         .         .         .         .         .      -159
TGGATTGCACGCTGCAATACTGACTGGCGCCGTGTGGCCGGGATCCCGTCACTTTCCACT

         .         .         .         .         .       -99
CTTGGTTTGCAACTTTTTTGTTCCTACTATTTCTTACGCCTCACCGGAGCTTTTTCTTCC

         .         .         .         .         .       -39
TCTCCCTTTCCCCTTTCCTGTTCTCAGTCATCTCAACTTCCAGTATTCCCTATACACCTT

         .         .         .       +1.         .        22
TGCGGTTTTAACGCTTCCTCATAAACCAATCAGTCAAAATGGGCTTCTCTGCCGGTATGT
                             ------------              =====
                                      MetGlyPheSerAlaG          5

         .         .         .         .         .        82
ATCCCACTCTCCTCTCCTCACCCACGCCATGATGCGCACGCGCTCTCTTTCTTTTATTGT

         .         .         .         .         .       142
TGTCTCGGCGGTCCGGCTCGTCAACGTCATCCGCCCGTCCGCCGGTCTTAGTTTCGCTGG

         .         .         .         .         .       202
TTGAGCCATTGGGTGTTCGCAATGTCCACATGAGTTGAAGAAGAGGCTACCACCCCTCGG

         .         .         .         .         .        62
TCACGGCTTCTTCAGCATCTCCATGGACAGGGCACCTCGAGCATGGCCTCGGTTTCCGAT

         .         .         .         .         .       322
CCCGACTCCGATGGTTGCGCGGCATTTTCTCCTTGTTTCGTAAACACGATCACTTGACGG

         .         .         .         .         .       382
AGCGAAATCGCGCCGTCATGGGAGATTGGCCACGTCGGCTTGAGGCCACGGAGCATGTCT

         .         .         .         .         .       442
TGCCCTACGACATACTTCCCTCACCACCATCTACCAGATCTCCAGCCATCAAGGTCTTGC

         .         .         .         .         .       502
TCCTGGACACCACGACCACGACAGTCGAGGCCGAGACTACCGAGATAATATCAGAATGAT
```

```
         .         .         .         .         .       562
CAGAGCCGCGCTTTGCTATCTTGCCGATAAACAGCCGTTCTAACCTAAAACTTGCGACCA

         .      □  .         .         .         .       622
GGTGATTCCAAGAAGGGTGCCAACCTCTTCAAGACCCGTTGCGCTCAGTGCCACACCCTT
 lyAspSerLysLysGlyAlaAsnLeuPheLysThrArgCysAlaGlnCysHisThrLeu  25

         .         .         .         .         .       682
GAGGAGGGCGGCGGCAACAAGATCGGCCCCGCTCTTCACGGCCTCTTCGGCCGCAAGACC
GluGluGlyGlyGlyAsnLysIleGlyProAlaLeuHisGlyLeuPheGlyArgLysThr  45

         .         .         .         .         .       742
GGCTCCGTCGACGGCTACGCCTACACCGATGCCAACAAGCAGAAGGGCATCACCTGGGAC
GlySerValAspGlyTyrAlaTyrThrAspAlaAsnLysGlnLysGlyIleThrTrpAsp  65

         .         .         .         .         .       802
GAGAACACTCTCTTCGAGTACCTCGAGAACCCCAAGAAGTACATCCCTGGTACCAAGATG
GluAsnThrLeuPheGluTyrLeuGluAsnProLysLysTyrIleProGlyThrLysMet  85

         .         .         .         .         .       862
GCCTTCGGTGGTCTCAAGAAGGACAAGGACAGGAACGACATCATCACGTACGTCATGCGC
AlaPheGlyGlyLeuLysLysAspLysAspArgAsnAspIleIleTh               100

         .         .         .         .         .       922
TGCTCCCCTATATTTGTCACTCAAAAAAAAATGCAAAGCTAACTCGATTTCACTCCCACA

         .         .         .         .         .       982
GCTTCATGAAGGAGGCTACTGCTTAAATGCAATCTGTTTGATGATGGGCGTTGTTCTCGA
 rPheMetLysGluAlaThrAlaEnd                                    108

         .         .         .         .         .      1042
GGAGTTATGGGACTGTATTAATAAAAAGGGAGATTTTTTTTTTGCATTAGACGGGCTTGG

         .         .
CACATCCCTCTTCGTTGTTGGACCA
```

Figure 16. Southern Blot of *Cyt-12-1* DNA Digested with Various Restriction Enzymes

Southern analysis of *N.crassa cyt-12-1* genomic DNA cut with the restriction endonucleases BamHI, EcoRI, HindIII, or HindIII and PstI. The probe was ^{32}P-labelled 3.9 kb HindIII-PstI fragment, isolated from pSP8. The marker used is lambda digested with HindIII and the fragment lengths are in kilobases.

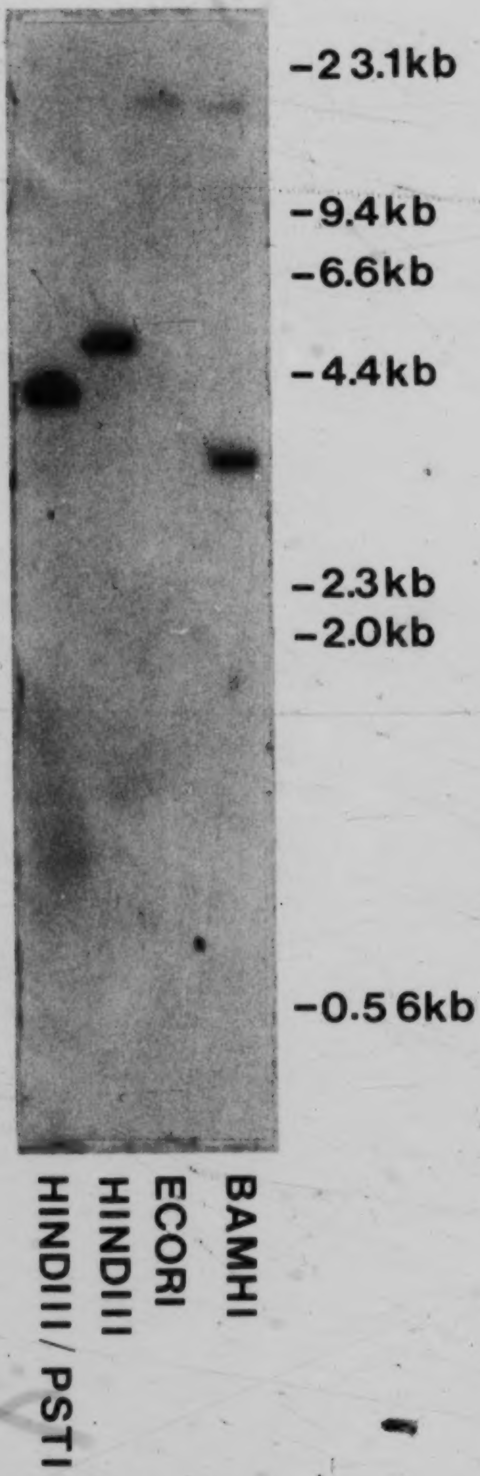

-2 3.1kb
-9.4kb
-6.6kb
-4.4kb
-2.3kb
-2.0kb
-0.5 6kb
HINDIII/ PSTI
HINDIII
ECORI
BAMHI

Figure 17. Partial Restriction Map and DNA Sequencing Strategy of the *Cyt-12-1* Mutant Allele

The arrows indicate the direction and extent of sequence from obtained by annealing various primers to the three phage clones containing either the 0.93 kb BamHI-HindIII, 3 kb BamHI-PstI, and 1.8 kb SalI-HindIII fragments from *cyt-12-1*, respectively. Four cytochrome *c* specific primers were synthesized (cyt*c*1, cyt*c*2, cyt*c*3, and cyt*c*4) and were used to obtain *cyt-12-1* sequence. The arrows specified by the numbers is sequence determined from the use of the specific primers. The asterisk indicates where the cyt-12-1 missense mutation is located in the cytochrome c gene. The heavy lines represent the exons of the cytochrome *c* gene, while the light lines represent the intervening sequences.

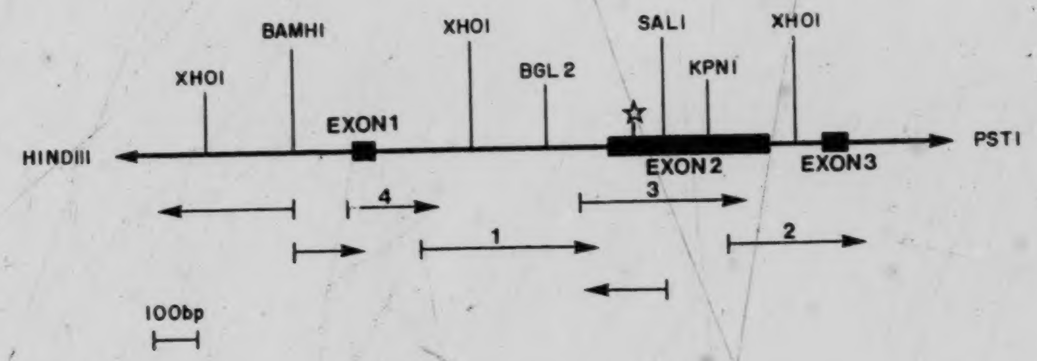

XHOI
BAMHI
XHOI
BGL 2
SALI
KPNI
XHOI
EXON1
HINDIII
PSTI
EXON2
EXON3
4
3
1
2
100bp

Figure 18.

The *Cyt-12-1* Missense Mutation

The nucleotide difference observed in the coding between the wild-type cytochrome *c* gene and *cyt-12-1*. The affected codon of *cyt-12-1* encodes an asparatic acid (GAT) instead of the wild-type glycine (GGT).

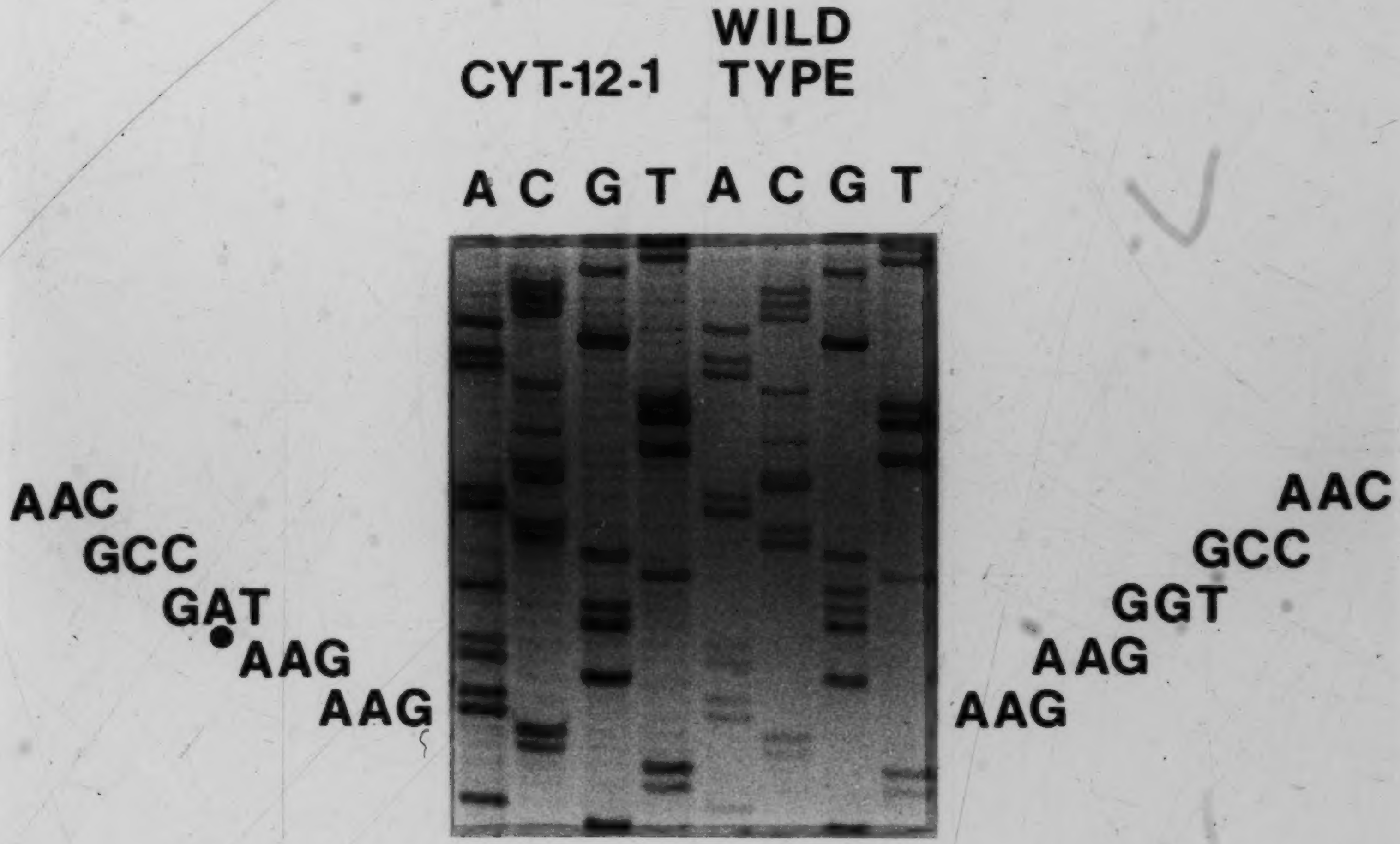

CYT-12-1
WILD TYPE
A C G T A C G T
AAC
GCC
GAT
●
AAG
AAG
AAC
GCC
GGT
AAG
AAG

Figure 19. Cloning and Partial Restriction Maps of pBEN1 and pBEN2

The 2.58 kb SalI-SalI fragment of pSV50, containing the benomyl resistant beta-tubulin gene, was cloned into SalI digested pUC19. The two different clones were designated pBEN1 and pBEN2. The arrows indicate the orientations of the ampicillin resistant gene (Ap^R) and the benomyl resistant form of the beta-tubulin gene (Bn^R).

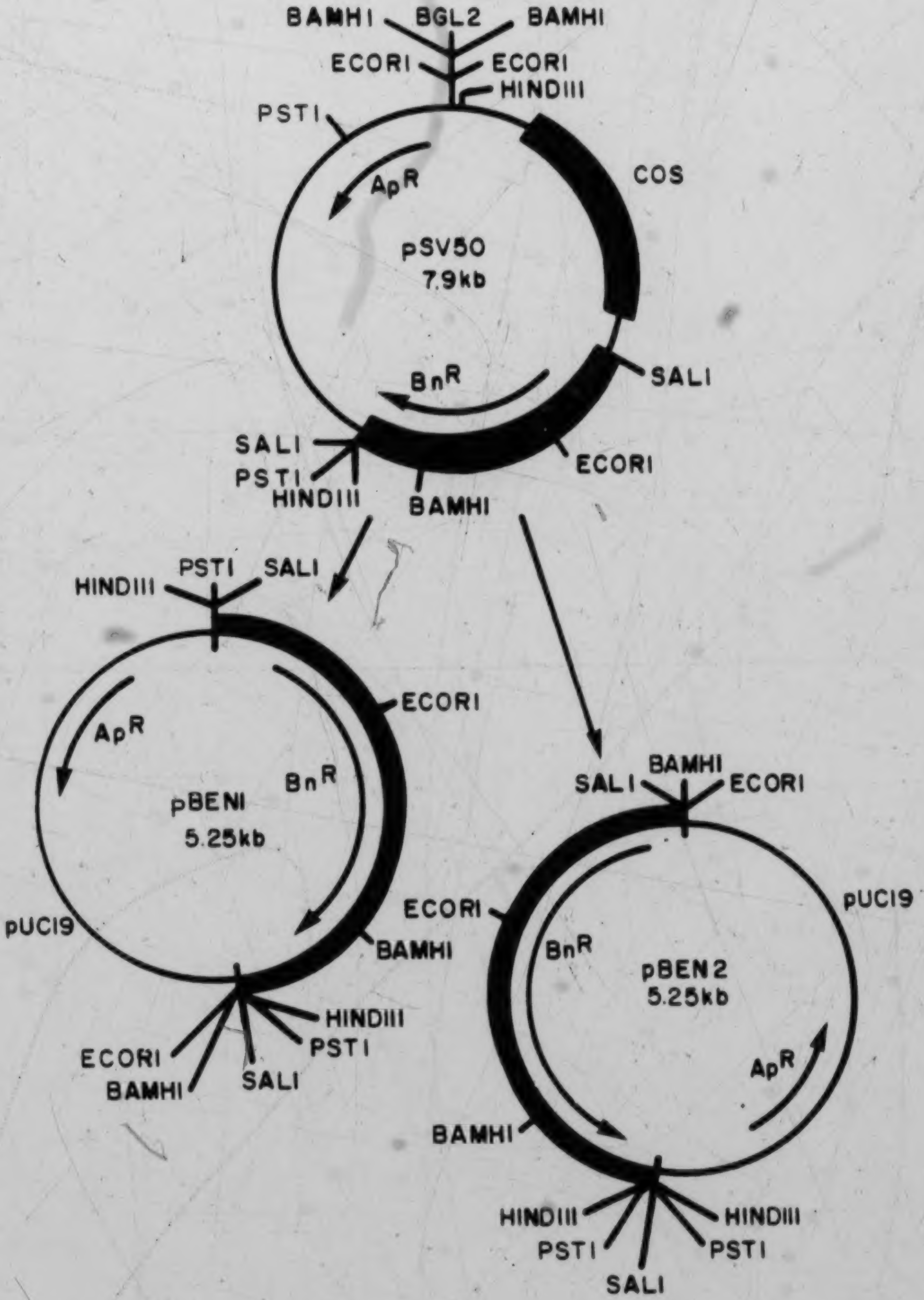

BAMHI
BGL2
BAMHI
ECORI
ECORI
HINDIII
PSTI
COS
ApR
pSV50
7.9kb
SALI
BnR
SALI
PSTI
HINDIII
BAMHI
ECORI
HINDIII
PSTI
SALI
ECORI
ApR
BnR
pBENI
5.25kb
pUC19
BAMHI
HINDIII
PSTI
ECORI
BAMHI
SALI
SALI
BAMHI
ECORI
pUC19
ECORI
BnR
pBEN2
5.25kb
ApR
BAMHI
HINDIII
HINDIII
PSTI
PSTI
SALI

Figure 20.
Northern Blot of Wild-type and *Cyt-12-1*
N.crassa RNA

Northern analysis of wild-type (NCN10) and *cyt-12-1* RNA by A) ^{32}P-labelled cytochrome *c* cDNA and by B) ^{32}P-labelled 2.58 kb SalI-SalI fragment containing the benomyl-resistant beta-tubulin gene. Lanes A, B, and C contain total RNA (37μg, 50μg, and 36μg, respectively) isolated from 3 different NCN10 (wild-type) RNA preparations. Lanes D, E, and F contain total RNA (30μg, 44μg, and 48μg, respectively) isolated from three different *cyt-12-1* total RNA preparations. Lanes G and H contain poly (A+) RNA isolated from separate *cyt-12-1* total RNA preparations (2.4μg and 2.9μg, respectively). Lanes I and J contain poly (A^+) enriched RNA (2.1μg and 2.2μg, respectively) from separate wild-type RNA isolates. The marker used during electrophoresis was the 9.5 kb single-stranded RNA ladder (BRL, Inc.).

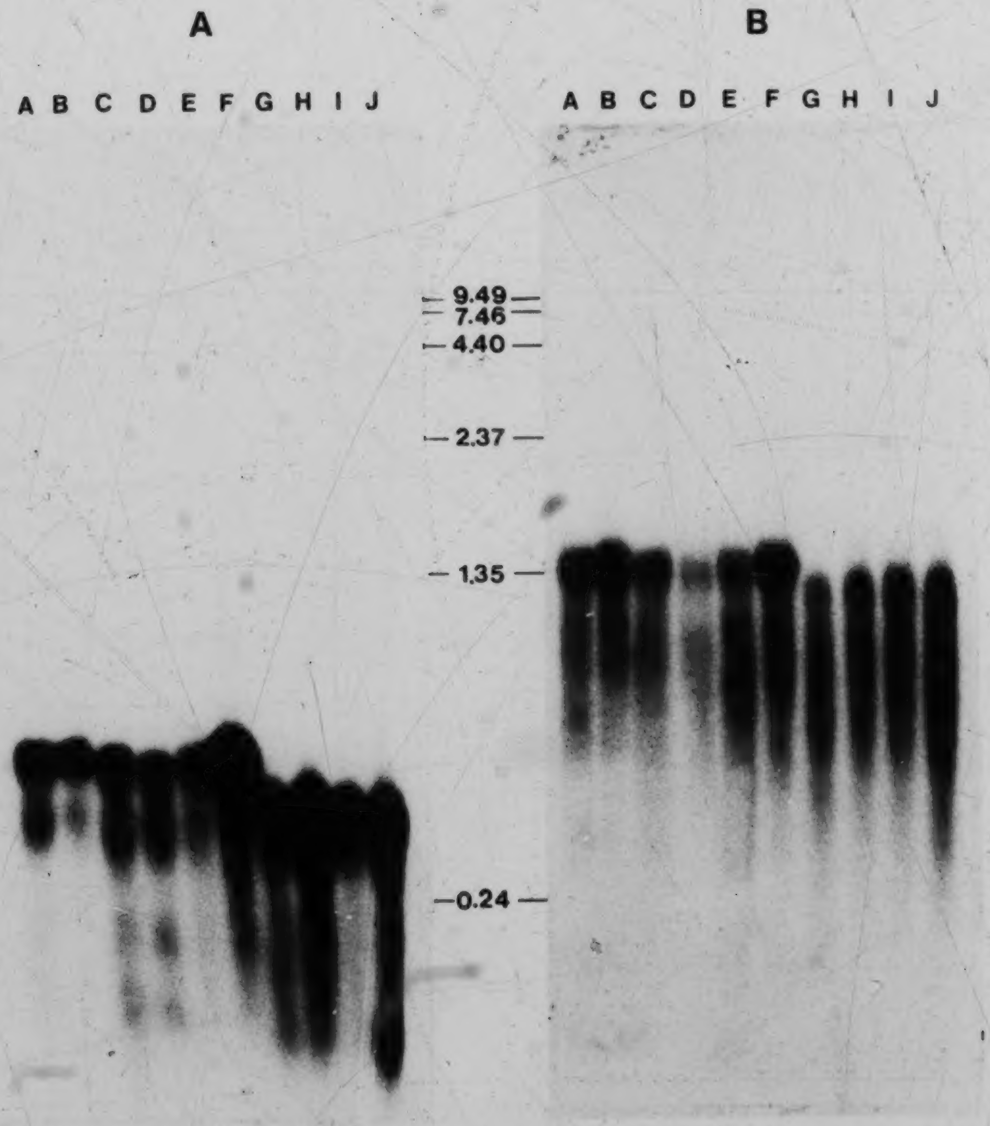

A
B
A B C D E F G H I J
A B C D E F G H I J
9.49
7.46
4.40
2.37
1.35
0.24

Figure 21. Immunoprecipitation of Wild-type (NCN10) Apocytochrome *c*

Rabbit reticulocyte *in vitro* translation reactions were programed with wild-type (NCN10) poly (A^+) RNA, using ^{35}S-methionine as the labelled amino acid. The *in vitro* translation products were electrophoresed on a 10% SDS discontinuous polyacrylamide gel. The lane was sliced into 1.1 mm gel slices. Slice zero corresponds to the bottom of the PAGE gel, which is backwards to normal convention. The gel slices were processed according to Materials and Methods. The counts per minute of ^{35}S for each individual gel slice is plotted on Figure 21.

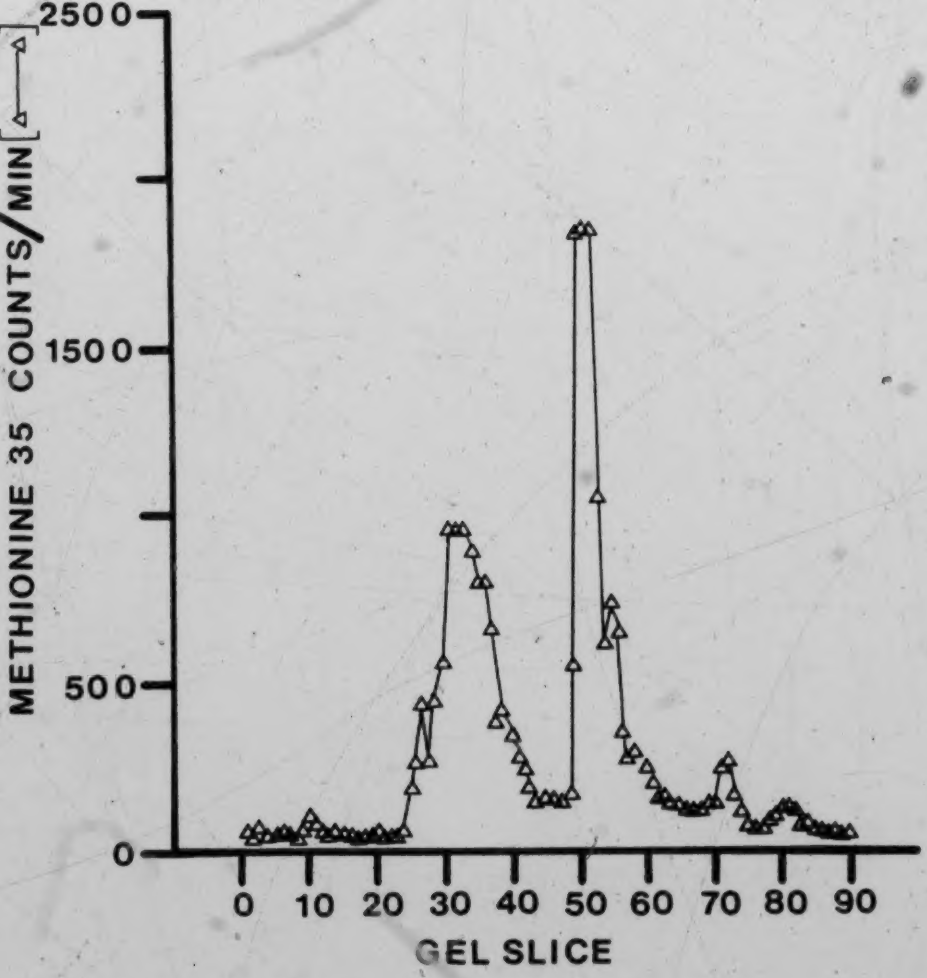

METHIONINE 35 COUNTS/MIN [△—△]
2500
1500
500
0
0
10
20
30
40
50
60
70
80
90
GEL SLICE

Figure 22. Immunoprecipitation of *Cyt-12-1* (NCN82) Apocytochrome *c*

Rabbit reticulocyte *in vitro* translation reactions were programed with *cyt-12-1* poly (A^+) RNA, using ^{35}S-methionine as the labelled amino acid. The *in vitro* translation products were electrophoresed on a 10% SDS discontinuous polyacrylamide gel. The lane was sliced into 1.1 mm gel slices. Slice zero corresponds to the bottom of the PAGE gel, which is backwards to normal convention. The gel slices were processed according to Materials and Methods. The counts per minute of ^{35}S for each individual gel slice is plotted.

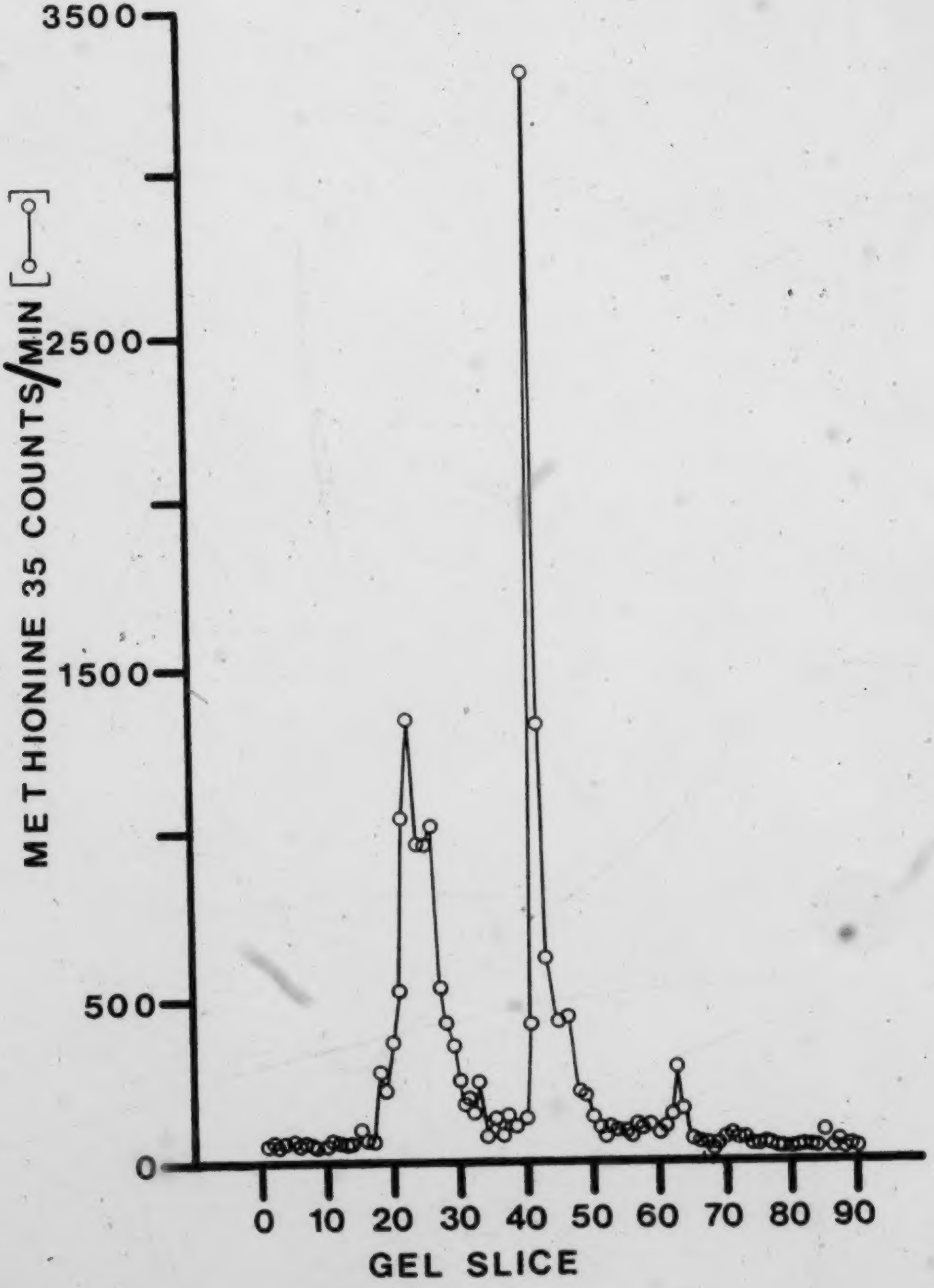

3500
2500
1500
500
0
METHIONINE 35 COUNTS/MIN [○—○]
0
10
20
30
40
50
60
70
80
90
GEL SLICE

Figure 23.
Flourogram of Holocytochrome *c* Immunoprecipitates From *Cyt-12-1* and Wild-type *N.crassa* Strains

Holocytochrome *c* was immunoprecipitated from *in vivo* labelled *cyt-12-1* and wild-type (NCN10) strains. *Cyt-12-1* cultures were labelled with ^{3}H-Leucine, while wild-type cells were labelled with ^{35}S-Methionine. Lane A contains holocytochrome *c* immunoprecipitates from *cyt-12-1*, Lanes B and C contain slightly different amounts of holocytochrome *c* immunoprecipitates from the wild-type strain. The protein standard used during electrophoresis is in kdal (Bio-Rad, Inc.). The time of exposure of the flourogram was 30 hours.

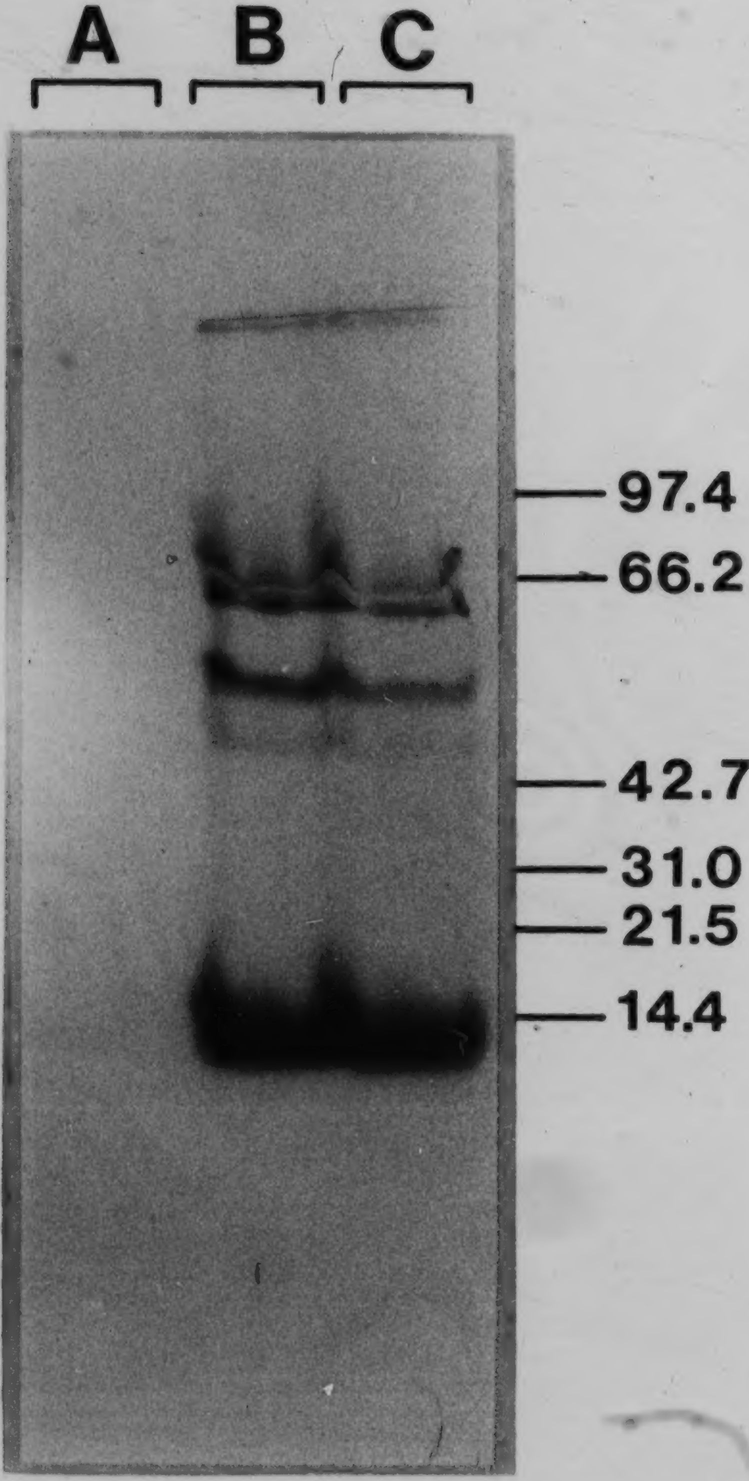
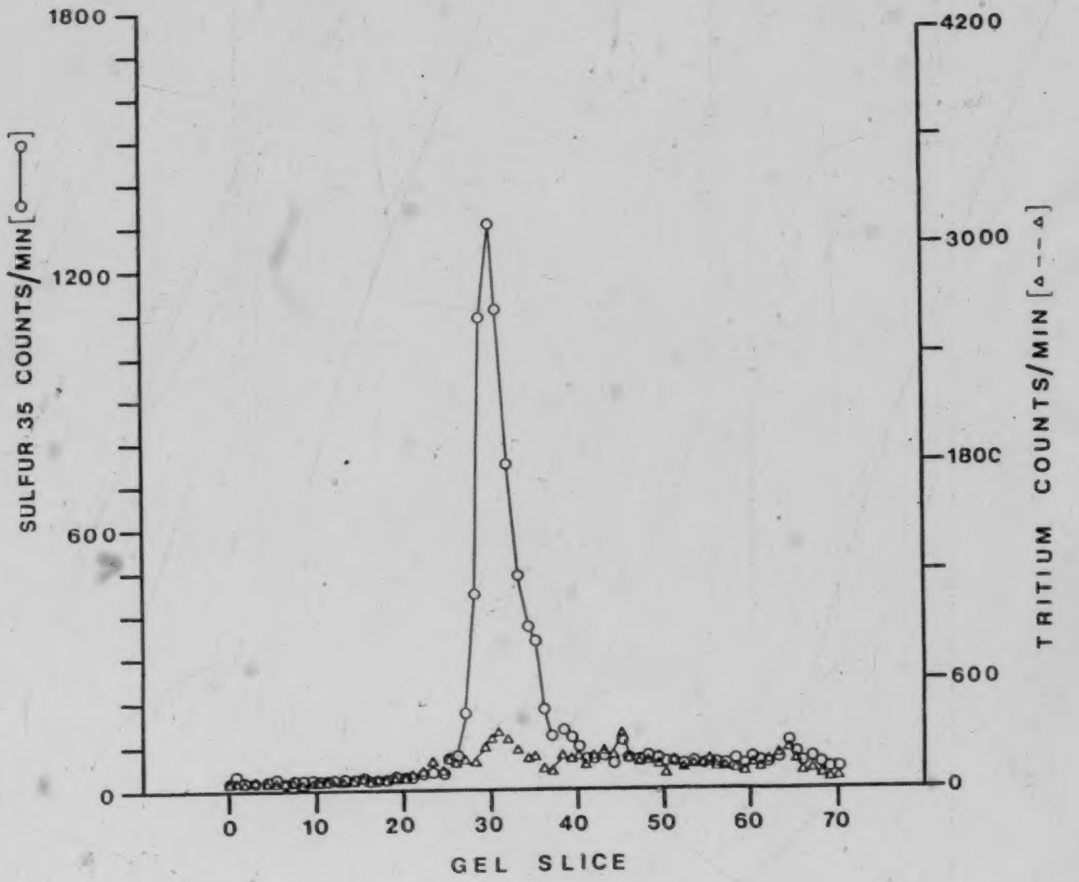

A
B
C
97.4
66.2
42.7
31.0
21.5
14.4

Figure 24. Holocytochrome *c* Immunoprecipitation Analysis of *Cyt-12-1* and Wild-type Mitochondria

Cyt-12-1 and wild-type (NCN10) cultures were differentially labelled with ^{3}H-Leucine and ^{35}S-Methionine, respectively. *Cyt-12-1* and wild-type mitochondria were mixed and holocytochrome *c* was immunoprecipitated by using specific antisera. The co-immunoprecipitates were electrophoresed on a 10% SDS discontinuous polyacrylamide gel. The lane was sliced into 1.1 mm gel slices and processed according to Materials and Methods. Slice zero corresponds to the bottom of the gel (i.e. high molecular weight). The cpm of ^{35}S and ^{3}H were determined and the counts of each isotope were plotted. The plot ratios reflect the fact that the cpm of solubilized cyt-12-1 mitochondria was approximately 2.5 fold more than the cpm of solubilized wild-type mitochondria used for the co-immunoprecipitation.


1800
1200
600
0
SULFUR 35 COUNTS/MIN [o—o]
4200
3000
180C
600
0
TRITIUM COUNTS/MIN [△--△]
0
10
20
30
40
50
60
70
GEL SLICE

Bibliography

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Appendix

I. Bacterial Media:

L-broth

(Lennox, 1955)

10 g	bacto-tryptone (Difco)
5 g	yeast extract (Difco)
5 g	NaCl
1 g	D-glucose

The pH was adjusted to 7.4 with 5N NaOH. The solution was made up to 1 l with water and sterilized by autoclaving for 25 minutes. Plating medium was made by adding 15 g Bacto-agar (Difco) to 1 l of the above medium prior to autoclaving. The culture conditions for selection of antibiotic resistance is given in the *E.coli* strains and culture conditions section.

Soft agar

10 g	Bacto-tryptone (Difco)
5 g	yeast extract (Difco)
5 g	NaCl
1 g	D-glucose
7 g	Bacto-agar

The solution was made up to 1 l with water and the pH was adjusted to 7.4 by adding concentrated NaOH. The solution was melted by heating, 3 ml of soft agar was dispensed to each tube and sterilized by autoclaving.

D.M. Salts & Thiamine & Glucose

(Davis and Mingioli, 1950)

50 ml	20X D.M. stock
1 ml	10% $MgSO_4$
1 ml	10mM thiamine

The solution was made up to 500 ml with water and sterilized by autoclaving for 25 minutes. Plating medium was made by adding 7.5 g Bacto-agar (Difco) to 500 ml of the above medium prior to autoclaving. A 1% (w/v) solution of sterile D-glucose (500 ml) was added when the above solution had cooled.

20X D.M. Stock

20 g	$(NH_4)_2SO_4$
60 g	KH_2PO_4
140 g	K_2HPO_4

This solution was made up to 1 l with water and 5 ml of chloroform was added as a preservative. This stock was stored at room temperature.

II. Neurospora Media and Solutions:

Vogel's Medium

(Davis and de Serres, 1970)

20 ml	50X Vogel's stock
1 ml	1X trace elements
1 ml	biotin solution
15 g	table sugar

Supplements were added as required. The solution was made up to 1 l with water and sterilized by autoclaving. Plating media was adding 15 g Bacto-agar (Difco) to the above medium prior to autoclaving.

0.5X Vogel's

10 ml	50X Vogel's stock
0.5 ml	biotin solution
0.5 ml	1X trace elements
7.5 g	table sugar

Supplements were added as necessary, but only 1/2 the amount added to Vogel's and viability media. The solution was made to 1 l with water before autoclaving.

50X Vogel's

(Davis and de Serres, 1970)

125 g	$Na_3citrate \cdot 2H_2O$
250 g	KH_2PO_4
100 g	NH_4NO_3
10 g	$MgSO_4 \cdot 7H_2O$
5 g	$CaCl_2 \cdot 2H_2O$

The first three ingredients were dissolved in 650 ml of water. The $MgSO_4$ and $CaCl_2$ were dissolved separately in 35 ml and 100 ml of water respectively. These two solutions were added sequentially to the first. When ingredients were completely dissolved, it was made up to 1 l with water. Chloroform (5 ml) was added as a preservative. The solution was stored at room temperature.

4X Trace Elements

(Davis and de Serres, 1970)

50 g	Citric Acid $1H_2O$
50 g	$ZnSO_4 \cdot 7H_2O$
10 g	$Fe(NH_4)2SO_4 \cdot 6H_2O$
2.5 g	$CuSO_4 \cdot 5H_2O$
0.5 g	$MnSO_4 \cdot 1H_2O$
0.5 g	H_3BO_3
0.5 g	$Na_2MoO_4 \cdot 2H_2O$
20 mg	$CoCl_2$

The solution was made up to 1 l with water. A 1X stock was made to use as a working solution. Both solutions were stored at 4°C.

Biotin Solution

(Davis and de Serres, 1970)

5 mg of biotin was dissolved in 400 ml of 50% (v/v) Ethanol and stored at 4°C.

Supplements

(Davis and de Serres, 1970)

L-amino acids and vitamins were added to a final concentration of 200 µg/ml and 10 µg/ml, respectively, when necessary.

Acid-Washed Sand

A large glass container 3/4 full with sand (sea sand or Ottawa sand) was filled with concentrated HCl and left overnight in the fumehood. Next morning, the acid was decanted and the sand was rinsed with water until the pH was neutral. The sand was dried in an oven, dispensed into containers and stored at 4°C.

Viability Plates

20 ml	50X Vogel's
1 ml	biotin solution
1 ml	Trace elements
15 g	agar

Supplements were added as required. The solution was made up to 900 ml with water. After autoclaving, 100 ml of 10X sugars solution was adding before pouring the plates.

10X Sugars

200 g	L-sorbose
5 g	D-fructose
5 g	D-glucose
2 g	myo-inositol

This solution was made up to 1 l with water and sterilized by autoclaving.

Top Agar

20 ml	50X Vogel's stock
1 ml	1X trace elements
10 ml	vitamins mix
15 g	agar
182 g	sorbitol

Supplements were added as required prior to making the solution up to 900 ml with water. After autoclaving, 100 ml of 10X sugars solution was added. For determining sphaeroplast viability and transformations no benomyl was added.

Vitamins Mix

30 mg	riboflavin
100 mg	thiamine
75 mg	pyridoxine
5 ml	p-aminobenzoic acid (2mg/ml)
200 mg	choline chloride
1 ml	folic acid
4 g	myo-inositol
0.5 g	adenine sulfate
0.5 g	uracil
0.5 g	L-methionine
0.5 g	L-arginine
1 g	L-Lysine
0.1 g	L-threonine
0.5 g	L-serine
0.2 g	L-homoserine
0.5 g	L-histidine
0.1 g	L-isoleucine
0.2 g	L-valine
0.2 g	L-leucine
0.5 g	L-proline

The riboflavin was dissolved in 100 ml of water with 4 drops of 5N NaOH before adding to the rest of the solution. After making up to 1 l with water, the solution was filter sterilized and stored in dark bottles at 4°C.

Benomyl Plates

Benomyl plates were prepared exactly like viability plates except that 2 ml of a benomyl solution (0.25 mg/l in 95% Ethanol) was added when the 100 ml of 10X sugars were added. The final concentration of benomyl was 0.5 µg/ml.

PMC

400 g	PEG-4000
4.2 g	MOPS (3-(N-morpholino)propanosulfonic acid)
50 ml	$CaCl_2$

The solution was made up to 1 l with water. The pH was adjusted to 6.3 and steilized by autoclaving.

III. DNA Sequencing Mixes and Solutions:

Regular Mixes

A mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
140 ul	dH_2O
1 ul	10 mM ddATP

C mix:

4 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
120 ul	dH_2O
2 ul	10 mM ddCTP

G mix:

40 ul	0.5 mM dCTP
4 ul	0.5 mM dGTP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
110 ul	dH_2O
4 ul	10 mM ddGTP

T mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM dGTP
4 ul	0.5 mM dTTP
40 ul	10X pol buffer
100 ul	dH_2O
8 ul	10 mM ddTTP

dITP mixes:

(Sanger *et al.*, 1982)

A mix:

20 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
20 ul	0.5 mM dTTP
20 ul	10X pol buffer
40 ul	dH_2O
0.25 ul	10 mM ddATP

C mix:

2 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
20 ul	0.5 mM dTTP
20 ul	10X pol buffer
60 ul	dH_2O
1.5 ul	10 mM ddCTP

G mix:

20 ul	0.5 mM dCTP
3 ul	0.5 mM dITP
20 ul	0.5 mM dTTP
20 ul	10X pol buffer
140 ul	dH_2O
1.5 ul	1 mM ddGTP

T mix:

20 ul	0.5 mM dCTP
100 ul	0.5 mM dITP
2 ul	0.5 mM dTTP
20 ul	10X pol buffer
60 ul	dH_2O
4 ul	10 mM ddTTP

Deazaguanine Mixes

(Barr *et al.*, 1986)

A mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM dITP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
140 ul	dH$_2$O
1 ul	10 mM ddATP

C mix:

4 ul	0.5 mM dCTP
40 ul	0.5 mM dITP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
120 ul	dH$_2$O
2 ul	10 mM ddCTP

G mix:

40 ul	0.5 mM dCTP
4 ul	0.5 mM c^7dGTP
40 ul	0.5 mM dTTP
40 ul	10X pol buffer
110 ul	dH$_2$O
4 ul	10 mM ddGTP

T mix:

40 ul	0.5 mM dCTP
40 ul	0.5 mM c^7dGTP
4 ul	0.5 mM dTTP
40 ul	10X pol buffer
110 ul	dH$_2$O
40 ul	10 mM ddGTP

Chase:

20 ul	10 mM dATP
20 ul	10 mM dCTP
20 ul	10 mM dGTP
20 ul	10 mM dTTP
120 ul	10 dH$_2$O

10X Pol Buffer

0.7 ml	1M Tris·HCl, pH 8.0
142 mg	$MgCl_2 \cdot 6H_2O$
1 ml	5M NaCl

The solution was made up to 10 ml with water.

Dye-Formamide Mix

10 mg	xylene cyanole FF (Kodak)
10 mg	bromphenol blue
400 ul	0.25 M EDTA, pH 8.0
9.5 ml	deionized formamide[1]

1. The deionized formamide was prepared as follows: To 100 ml formamide , 10 g of mixed-bed resin (Bio-Rad AG 501-X8, 20-50 mesh) was added. This mixture was stirred for 30 minutes. The formamide was decanted into a clean beaker and 10 g of mixed-bed resin was further added. After mixing for 30 minutes, the formamide was decanted into a clean container. The deionized formamide was aliquoted into 10 ml disposable polypropylene tubes and stored at -20°C.

40% Acrylamide

380 g	acrylamide
20 g	bis-acrylamide
500 ml	dH_2O

The solution was made up to 1 l after dissolved in the fumehood. This solution was stored in the dark at 4°C.

IV. Hybridization and Probe buffers:

50X Denhardt's

5 g	ficoll
5 g	polyvinylpyrrolidone
5 g	BSA

This was made up to 500 ml with water and stored at -20°C.

Hybridization buffer

(adapted from Wahl *et al.*, 1979)

50 ml	50X Denhardt's
12.5 ml	20X SSC
25 ml	1 M $NaPO_4$, pH 6.5
5 ml	10% SDS (w/v)
12.5 ml	10 mg/ml salmon sperm DNA[1]
250 ml	formamide (non-deionized)
145 ml	dH_2O

[1] Salmon sperm DNA was prepared as follows: The DNA (Sigma Type-III sodium salt) was dissolved in water at a concentration of 10 mg/ml, on a magnetic stirrer for 2 hours at room temperature. The DNA was sheared by several 10 second intervals of sonication.

The hybridization buffer was heated for 5 minutes in a boiling-water bath and chilled quickly on ice prior to use. This solution was stored at -20°C.

OLB Buffer

OLB was prepared by mixing solutions A:B:C in a ratio of 100:250:150 and stored at -20°C.

Solution O:

1.25M	Tris·HCl, pH 8.0
0.125M	$MgCl_2$

This solution was stored at 4°C.

Solution A:

1 ml	solution O
18 ul	Beta-mercaptoethanol
5 ul	0.1 M dATP, 3 mM Tris·HCl, pH 7.0, 0.2 mM EDTA
5 ul	0.1 M dGTP, 3 mM Tris·HCl, pH 7.0, 0.2 mM EDTA
5 ul	0.1 M dTTP, 3 mM Tris·HCl, pH 7.0, 0.2 mM EDTA

This solution was stored at -20°C.

Solution B:

2 mM	Hepes, pH 6.6

This solution was titrated with 4M NaOH and stored at 4°C.

Solution C:

90 OD units/ml	Hexadeoxyribonucleotides (P-L Biochemicals)
3 mM	Tris·HCl, pH 7.0
2 mM	EDTA

This solution was stored at -20°C.

V. Northern and RNA buffers:

Part A

8.2 g	NaAcetate
0.29 g	EDTA
40 g	SDS

This was made up to 1 l with water and the pH was adjusted to 5.0. The solution was DEPC-treated prior to autoclaving and stored at room temperature.

Part B

500 ml	water-saturated phenol
500 ml	chloroform/iso-amyl alcohol (24:1), (v/v)

This solution was stored at 4 C.

4 M NaAc Solution

164 g	NaAcetate
1.45 g	EDTA

This was made up to 500 ml with water and the pH was adjusted to 6.0. The solution was DEPC-treated prior to autoclaving and stored at room temperature.

Northern Hybridization buffer

This solution is the same as hybridization buffer except that deionized formamide and 1/2 the amount of non-homologous DNA are used.

10X MOPS/EDTA

0.2 M	MOPS (3-(N-morpholino) pro-panesulfonic acid
50 mM	NaAcetate
10 mM	EDTA

The pH of this solution adjusted to 7.0. The solution was DEPC-treated prior to autoclaving.

Electrophoresis Sample Buffer

0.75 ml	deionized formamide
0.15 ml	10X MOPS/EDTA
0.24 ml	deionized 37% (v/v) formaldehyde
0.1 ml	DEPC-treated water
0.1 ml	glycerol
0.08 ml	10% (w/v) bromphenol blue

Deionized 37% (v/v) Formaldehyde

This solution was prepared the same as deionized formamide except that is was stored in a dark bottle at room temperature.

VI. Miscellaneous Solutions:

30% Acrylamide

290 g	acrylamide
10 g	bis-acrylamide
500 ml	dH_2O

After dissolved in the fumehood, this solution was made up to 1 l with water and stored at 4°C.

Salt-saturated Isopropanol

100 g	NaCl
10 ml	1M Tris·HCl, pH 7.3
50 ml	dH_2O
500 ml	isopropanol

The solution was mixed thoroughly and the phases were allowed to separate. The upper phase was used to extract ethidium bromide from aqueous solutions.

Protease K Solution

60 mg of protease K (Sigma) was dissolved in 10 ml of 100 mM Tris·HCl, pH 7.3 , 150 mM NaCl, 1 mM EDTA. After incubated in a 37°C water-bath for 60 minutes, 10 ml of glycerol was added. The solution was stored at -20°C, after the solution was mixed thoroughly.